VARIATION IN GENOTOXICITY DUE TO DIFFERENCES IN METABOLIC ACTIVATION

STUDIES WITH ISOLATED HEPATOCYTES OF DIFFERENT SPECIES INCLUDING MAN

BY
JOHN NEIS

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. J. H. G. I. GIESBERS VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 17 JANUARI 1986 DES NAMIDDAGS TE 2 UUR PRECIES

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aan mijn ouders

aan Anita

aan Hennie

Paul

"I do not think we can rely on extrapolations of data on chemical carcinogenesis from experimental animals to humans, no matter how sophisticated or plausible these extrapolations may seem, until we know more about chemical carcinogenesis in humans"

Dr. James A. Miller

CONTENTS

Biological effects of genotoxic chemicals

Body-foreign chemicals can possess intrinsic electrophilic properties or they can be activated into compounds with electrophilic properties. This process of activation either proceeds spontaneously, i.e. via a chemical reaction, or by means of enzymes.
Electrophiles are capable to attack selectively nucleophilic sites of cellular constituents with vital importance, like lipids, proteins and nucleic acids. These interactions produce so-called "chemical-lesions", that can result in several disfunctions leading for instance to decreased cell-viability or even to necrosis and tissue degeneration (Mitchell, 1973; Lenders, 1983). When such electrophilic agents react with selective nucleophilic sites in DNA, e.g. at the N-7, C-8 and O-6 of guanidine, the induced lesions in the DNA may result in cytotoxic, mutagenic, carcinogenic or possibly teratogenic effects (Bora, 1982; Ehling, 1983; Schreiner, 1983; Sobels, 1973; Zeller, 1975) In some cases, cytogenetic changes like chromosomal aberrations, are also attributed to prior DNA-damage by genotoxic chemicals (Bora, 1982; Ehling, 1983; Sobels, 1973). Chemicals that do not possess electrophilic properties, but are capable of interacting with the DNA in other ways, e.g. acridine intercalation between adjacent nucleotide-bases, can also induce DNA lesions, thereby leading to genotoxic effects.
Induced lesions in the DNA can be found in somatic as well as in germ cells. If the error occurs in a somatic cell, the result may be cell death or the formation of daughter cells with altered genes or in certain cases tumors. In case of a germ cell, transmission of the error to sequent generations may occur, leading to early spontanous abortions, inborn errors of metabolism, or other congenital defects which might be inheritable if fertility is not affected (Hook, 1982).

Well-known inherited diseases like hemophilia and various types of anaemia are generally connected with the existence of naturally occurring gene mutations (Carter, 1982). It is likely to assume that exposure to genotoxic agents will increase these natural hazards and may even induce new diseases.
Indeed, interactions between electrophiles and DNA may initiate a broad spectrum of diseases. In most studies of the present thesis however, main attention has been paid to their mutagenic properties.

Chemical mutagenesis and carcinogenesis

The mechanisms involved in normal cells becoming malignant are still uncertain. However, the so called somatic mutation hypothesis, which is based on changes in genetic structure, has been accepted by many investigators to explain the primary events in chemical carcinogenesis. This theory, that mutation in somatic cells may lead to cancerous changes, was first suggested by Bauer (1928).
Evidence in support of this hypothesis has been provided by, among others, the classical work on the heritable disease xeroderma pigmentosum (Cleaver, 1969; Röhrborn, 1976). When skin cells of people suffering from this disease are irradiated with UV-light, defective DNA-repair (resulting in a high mutation rate) and a high frequency of malignant transformations are observed in these cells. It has been suggested that a defect in the code for an endonuclease (involved in DNA-repair) is responsible for this failure to repair the UV-damaged DNA. The theory of Bauer to explain neoplasia had a serious limitation. It did not fit in with the observation that the state of established neoplasia is often remote from the initiating mutational event by a substantial time interval, which in some cases can span almost half the life-time of the host.
It is now generally accepted that the process of carcinogenesis is a multi-step process, which may be divided into two main

steps, namely initation and promotion (Scribner, 1978; Slaga, 1980). Initiation is thought to be the primary event, which triggers the onset of the process of neoplasia. This may be the interaction of a DNA-damaging chemical with critical sites in the DNA. It is thought that the succeeding chain of events leading to the establishment of various malignant diseases, needs a second trigger, referred to as the promotion step. Many mutagenic agents have been shown to possess initiating properties. Several major studies have shown that there is a connection between mutagenic activities and carcinogenic activities of chemicals, with correlations ranging from about 67% to about 90% (Kawachi, 1980; McCann, 1975; Rinkus, 1979). This relationship has inspired the use of short-term mutagenicity assays in the detection of the potentially carcinogenic properties of chemicals.

Metabolic activation and inactivation

The metabolic transformations of body-foreign chemicals are divided into two types of reactions, phase I and phase II reactions (Sundberg, 1980). Phase I reactions, mainly concern oxidative processes, e.g. hydroxylations, epoxidations, oxidative dealkylations and deaminations, and to a lesser extent reductive and hydrolytic processes. Phase II reactions are involved in conjugations, for instance resulting in the synthesis of glucuronides, sulfates, acetates, and glutathione conjugates. Mostly, conjugation reactions follow phase I reactions, however the reverse also occurs.

Many genotoxic chemicals which are unreactive as such can be biotransformed, predominantly by phase I processes, into highly reactive products, capable of interacting with nucleophilic centers (Wright, 1980). Formerly, phase II reactions were thought to be mainly involved in detoxification processes. However, it becomes more and more evident that also conjugation reactions, e.g. sulfation and acetylation are involved in the

activation processes (Irving, 1979). Even conjugation with glutathione can lead to the formation of highly reactive products. For instance, glutathione can accelerate the alkylation of DNA by compounds such as 1-nitroso-1-methyl-3-nitrosoguanidine and nitrosomethylurethane, by reacting with these compounds, causing their decomposition into highly alkylating intermediates (Wheeler, 1972). Another example is the increased electrophilicity of 1,2-dihalogen alkanes by gluthatione conjugation (van Bladeren, 1980).

The ability of genotoxic chemicals to exert their genotoxic activity is strongly dependent on the relative rates of activation and deactivation reactions, which determine the steady-state levels of the ultimate genotoxic intermediates and consequently the degree at which chemical lesions are induced. Any interference in the various enzyme activities involved in the toxification and detoxification process, will result in a change in concentration of the ultimate genotoxic metabolites and may lead to an altered ultimate genotoxicity.

Many studies have provided evidence for the existence of considerable species-, strain-, sex- and interindividual variations in the metabolism of body-foreign chemicals (Conney, 1974; ECETOC, 1982; Nebert, 1983; Harris, 1984; Juchau, 1983; Kato, 1979). In recent years, using advanced techniques for the isolation and purification of isoenzymic forms of drug-metabolizing enzymes, and the observation of differential immunologic cross-reactivity, substrate specificity and amino acid sequences of these purified forms, insight in such variations in biotransformation is increasing (Guengerich, 1979; Lu, 1980; Boobis, 1984; Davies, 1984; Hines, 1984; Coon, 1984).

Many environmental chemicals are known to be capable of induction or inhibition of the metabolism of xenobiotics, probably by changing qualitatively and/or quantitatively the isoenzyme composition of the enzymes involved. Some well known modifiers are: phenobarbital, DDT, 3-methylcholanthrene, certain polychlorinated biphenyls, certain steroids, cigarette-smoke condensate and alcohol. The influence of diets on the metabolism of foreign compounds may be examplified by the observation of

Hietanen and Vainio (1973), that by feeding rats with a guinea pig diet, rat small-intestine benzo(a)pyrene hydroxylase activity could be 10-fold enhanced, reaching the same level as was measured in the guinea pig.

As we have genetically determined colour of hair and eyes, each individual has also a unique genetically determined pattern of enzyme activities concerned in the biotransformation of body-foreign chemicals. Because of this there are large genetic differences in the biotransformation of xenobiotic chemicals, that are supposed to result in important variations in risk towards drug-toxicity, mutation, certain types of cancer and birth defects (Omenn, 1984). In humans genetically controlled polymorphisms of N-acetylation of isoniazid and hydrolysis of succinylcholine have been known for many years. Polymorphisms of oxidative metabolism, involving cytochrome-P450 enzymes, however, have been recognized only recently among human populations (Omenn, 1984). Recently, particular attention has been paid to the genetic control of the inducibility of xenobiotic-metabolizing enzymes in animals as well as in humans. A wellknown example, is the Ah-locus that regulates the responsiveness of cells to various inducers belonging to the polycyclic aromatic compounds, such as 3-methylcholanthrene (Nebert, 1983). Several studies have indicated that a cytosolic receptor, capable of binding with high specificity certain polycyclic aromatic compounds, is produced by these Ah-genes. It is suggested, that when a cell is exposed to such inducers, the inducer-receptor complex formed activates certain other genes in the nucleus that regulate the biosynthesis of certain xenobiotic-metabolizing enzymes, e.g. certain cytochrome-P450 isoenzymes. Consequently, a change in the critical balance between toxification and detoxification will occur, which may result in crucial changes in the (geno)toxicity of certain xenobiotics. There is substantial evidence now that in many animal species as well as in humans, genetically determined differences in the concentration of this receptor exist, which lead to the differences in inducibility. It is most likely that these or such like genetic differences in inducibility play an

important role in the variations in susceptibility towards the potential (geno)toxic activity of certain body-foreign chemicals. In addition it should be realized that the interaction of ultimate reactive intermediates with critical targets not only depends on the steady-state concentration and chemico-physical properties of the reactive intermediates but also on the susceptibility of the targets. Possibly this susceptibility may also be subjected to species-, strain-, sex- and even interindividual differences.

Isolated hepatocytes in in-vitro genotoxicity studies.

The liver is the predominant organ involved in the metabolism of body-foreign chemicals. Within the liver the xenobiotic-metabolizing enzymes are concentrated mainly in the parenchymal cells, i.e. the hepatocytes, that comprise about 90% of the total liver mass and about 70% of the total amount of liver cells (Groothuis, 1982).

Intact hepatocytes can be isolated from liver tissue most properly by a perfusion technique using enzymatic (collagenase) digestion. With small animals, like rodent species, the perfusion technique can be applied to the whole liver, however in larger animals and in man, it is more suitable to perfuse only pieces of the liver.

The isolated hepatocytes can be used either immediately as suspension, in short-term experiments, or they can be used as primary cultures for rather long-term studies (several days or weeks). In the latter case the isolated hepatocytes have to be brought into a primary culture, under special conditions to enable the hepatocytes to survive this period in a non-proliferating state. Both systems have their advantages and shortcomings when used in in-vitro genotoxicity studies.

Freshly isolated hepatocytes may have a "shock" status because they have to adapt to the suboptimal conditions of their new environment. This may be examplified by an increased excretion

of proteins by these cells compared to the in-vivo situation (Princen, 1984). It is stated that hepatocytes cultured for about 24 hours have adapted and do not suffer from this aspect. Hepatocytes maintained as primary cultures, however, show several morphologic changes and a rapid decline in their biotransformational capacities, e.g. cytochrome-P450 catalyzed activities. Certain cytochrome-P450 isoenzymes are known to disappear although the total level of cytochrome-P450 may appear to be normal. This may be due to enhanced synthesis of other isoenzymic forms of cytochrome-P450 during culture. Several attempts have been made to artificially overcome the loss of certain enzyme activities. For instance, in order to preserve the "phenobarbital type P450" levels in culture, phenobarbital was incorporated in the culture medium as inducer (Blaauboer, 1985). The results of such approaches (although promising) seem stil far from ideal, because phenobarbital can also induce other enzymes (changing the metabolic profile as observed in-vivo), and may cause many other modifications to the cell, such as endoplasmatic reticulum proliferation, increased nuclear ploidy etc (Bridges, 1980). The dissimilar distribution of drug-metabolizing enzymes among hepatocytes in the liver in-vivo, e.g. centrilobular hepatocytes compared with periportal hepatocytes, is also present in a suspension of freshly isolated hepatocytes (Groothuis, 1982; Jungermann, 1982; Sweeney, 1981). However, it appeared that hepatocytes in primary culture for a longer period of time lost this heterogeneity.

The value of freshly isolated hepatocytes for the assessment of the metabolite profile of several body-foreign chemicals and the overall biotransformational rate has been demonstrated extensively during the last 15 years (McMahon, 1980; Brouns, 1981; Bridges, 1980; Roberfroid, 1980; Gould, 1983). In addition, isolated hepatocytes showed to be appropriate in models for the study of inhibition and/or induction of enzyme activities because of the possibility to control precisely the time and degree of exposure to xenobiotic chemicals.

Recently, studies with isolated hepatocytes were extended to more detailed investigations of the relationship between metabolic

aspects and primary effects of genotoxic compounds (Brouns, 1981; present thesis). In this respect the use of intact hepatocytes instead of the commonly used subcellular liver preparations, e.g. the S9 (9000 xg supernatant fraction), offers the opportunity of having the xenobiotic-metabolizing enzymes in relative proportions and spatial relationships resembling the in-vivo situation. Especially the natural balance between activating and detoxificating reactions, which determines the concentrations of the reactive intermediates, may be properly preserved. Moreover, freshly isolated hepatocytes are capable to synthesize and recycle most of the cofactors needed for the optimal function of the activating and deactivating enzymes, whereas S9 fractions can only be enriched artificially with cofactors.

As far as the endpoint of genotoxicity is concerned, various possibilities can be examined. Freshly isolated hepatocytes may be used in combination with a extra cellular response system, e.g. several genetically defined strains of Salmonella typhimurium, or on the other hand, the response system may be embodied within the hepatocyte, e.g. DNA-excision repair. The experimental set-up of these different approaches is outlined in Figure 1.

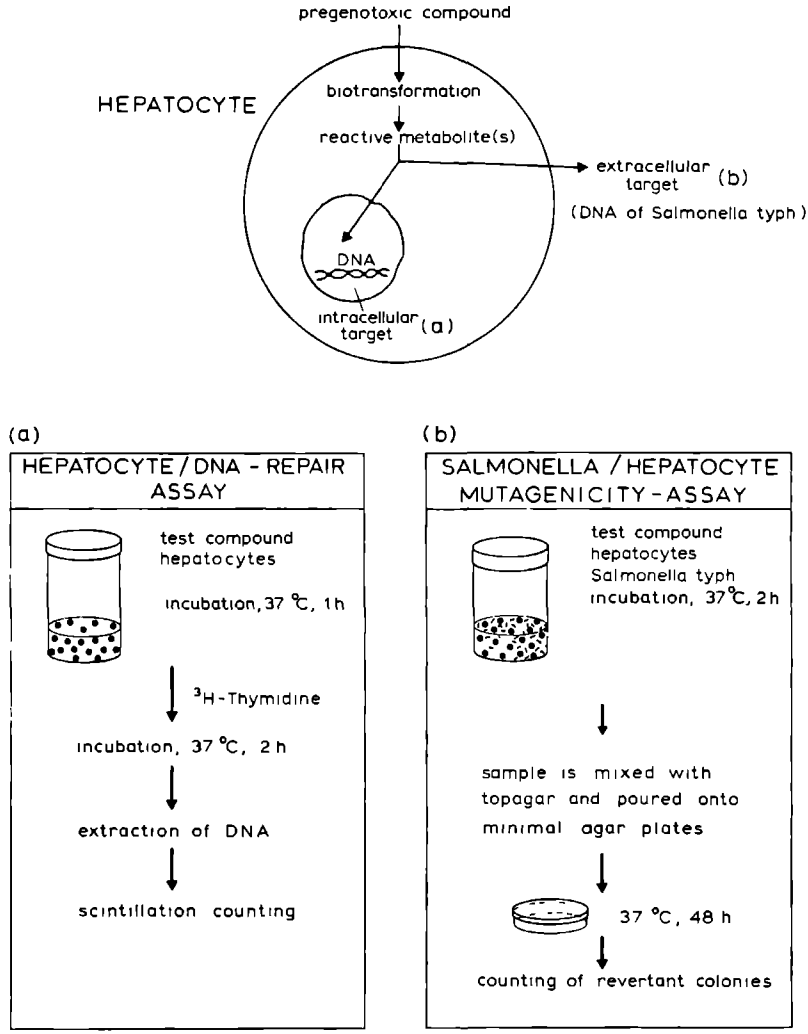

Fig. 1. A schematic representation of the hepatocyte/DNA-repair assay and the Salmonella/hepatocyte mutagenicity assay. In the Salmonella/hepatocyte assay, hepatocytes are used as metabolic system, and the reaction of reactive metabolites with the prokaryotic DNA of Salmonella typhimurium, mutating these histidine-auxotrophic bacteria back to histidine-autotrophy, is used as endpoint of genotoxicity. In the hepatocyte/DNA-repair assay, hepatocytes are used on one hand as the metabolizing system and on the other hand hepatocellular DNA-excision repair, evoked by adduct formation of reactive metabolites with the hepatocellular DNA, is used as endpoint of genotoxicity. DNA-excision repair is assayed via the measurement of 3H-TdR-incorporation into the hepatocellular DNA.

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The inter- and intraspecies variations in metabolic activation described in the previous chapter can be considered as important factors leading to differences in susceptibility to genotoxic chemicals. These differences make it very difficult to extrapolate genotoxicity data obtained with animal based studies to the human being.
In addition, there is concern about the limited predictive value of results of in-vitro studies on metabolic activation and genotoxicity. In this respect, for instance, there is doubt about the value of short-term in-vitro assays to identify potential carcinogens, in most of which subcellular liver fractions (S9) are used as metabolic system.

The investigations of the present thesis primarily have the objective to gain more insight in the mechanisms underlying the differences mentioned above. A better understanding of this matter would help us in future to develop more reliable methods for the indentification of genotoxic chemicals and to make more justified estimations of risks to humans.
The experimental approach of the present thesis mainly concentrates on:
- in-vitro/in-vivo differences.
- interspecies differences, in particular between laboratory animals and man.
- interindividual differences.

These problems were encountered by means of model studies with suspensions of freshly isolated hepatocytes derived from different species.
The most relevant points that were studied are these:
- differences in metabolic activation between the 9000 xg liver supernatant fraction (S9) and isolated intact hepatocytes (chapter III, IV, VII, IX).
- the influence of modifiers of metabolism (i.e. ethanol,

paraoxon, and the cofactor acetyl-CoA) on the genotoxicity certain chemicals (chapter III, IV, VI, X).
- differences in metabolic activation between isolat hepatocytes of different species including man (the species main interest) (chapter III-IX.)
- interindividual differences between human subjects studied wi isolated hepatocytes (chapter VII, VIII, IX).

It has to be realized that not only DNA can serve as a targ molecule for electrophiles. Many other nucleophilic sites can attacked. One particular example was studied in the prese investigation, namely the species-dependent difference susceptiblity towards electrophilic agents of SH-groups hemoglobin (chapter XI).

The results of these investigations are described in detail the chapters III-XI (consisting of prepublished or submitt papers) and are generally discussed in chapter XII.

Chapter III

MUTAGENICITY OF BENZIDINE AND 4-AMINOBIPHENYL AFTER METABOLIC ACTIVATION WITH ISOLATED HEPATOCYTES AND THE 9000 XG LIVER SUPERNATANT FROM RAT, HAMSTER AND GUINEA PIG

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SUMMARY

The mutagenicity of benzidine and 4-aminobiphenyl towards Salmonella typhimurium strain TA1538 was measured in the presence of isolated hepatocytes from rat, hamster and guinea pig. The mutagenic potency of these compounds was also assayed with S9 (9000 xg supernatant) prepared from disrupted hepatocytes. The influence of acetyl-CoA, the cofactor for the acetylation reaction, on the mutagenicity of these arylamines was investigated.

For all three animal species it was found that the mutagenicity of benzidine is higher with intact hepatocytes than with S9 prepared from disrupted hepatocytes. Addition of acetyl-CoA to the S9 fraction increased the mutagenicity of benzidine. In contrast to benzidine , the mutagenicity of 4-aminobiphenyl appeared to be lower with hepatocytes than with S9. Addition of acetyl-CoA to the S9 fraction decreased the mutagenicity of 4-aminobiphenyl.

The mutagenic potency of 4-aminobiphenyl was almost equal in the presence of the liver preparations from the three different species, whereas obvious species differences were seen with benzidine.

INTRODUCTION

Responses to toxic substances are markedly influenced by interspecies differences. The basis for these variations is thought to be primarily the difference in the nature and extent of the biotransformation. This appears to be the case particularly when the metabolic pathways are very complex.

Many chemical carcinogens, including certain arylamines, require metabolic activation to form reactive metabolites. Differences in activities of the enzymes necessary for the biotransformation can partly account for tissue and species differences in

genotoxicity of chemical compounds. For example the dog, lacking the N-acyltransferase activity involved in the metabolism of arylamines, develops only bladder tumors after receiving an unacetylated arylamine. Administration of the acetylated derivative results in liver and bladder tumors (Irving, 1979). It thus seems that acetylation is important for the formation of liver tumors by arylamines.

Previous investigations have shown that isolated hepatocytes can be considered as a valuable tool for the study of the biotransformation of genotoxic agents, particularly those compounds that undergo complex reaction sequences (Billings, 1977; Bock, 1976; Bos, 1982 and 1983; Glatt, 1981; Green, 1977). In the present study we compared the mutagenicity of two structurally related carcinogenic arylamines, benzidine and 4-aminobiphenyl, in the Salmonella/hepatocyte assay using isolated hepatocytes of rat, hamster and guinea pig, paying particular attention to the role of acetylation in the biotransformation of these compounds.

MATERIALS AND METHODS

Chemicals

Benzidine was purchased from Merck (Darmstadt, FRG). 4-Aminobiphenyl was from Aldrich Europe (Beerse, Belgium). D-Biotin, L-histidine-HCl, collagenase type 1, nicotinamide adenine dinucleotide phosphate disodium salt (NADP) and glucose-6-phosphate (G-6-P) dipotassium salt were obtained from Sigma (St. Louis, Missouri, USA). Acetyl-CoA was obtained from Boehringer (Mannheim, FRG). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain). All other chemicals used were of high purity.

Animals

The species of animals used in this study were as follows: male Wistar rats (app. 200 g), male albino guinea pigs (app. 400 g) and male golden Syrian hamsters (app. 90 g).

Preparation of isolated hepatocytes

The procedure was based on the methods described by Berry and Friend (1969) and by Seglen (1973), with some modifications. All manipulations were performed under sterile conditions. Before the procedure, the animals were anesthetized by an i.p. injection of sodium pentobarbital (60 mg/ml distilled water); 0.2 ml for rats, 0.7 ml for guinea pigs and 0.1 ml for hamsters. After pre-perfusion (15 min for rat and guinea pig, and 20 min for hamster) of the liver with a Ca2+-free HEPES buffer (pH 7.4), this buffer was replaced by a collagenase-containing HEPES buffer (for rat and guinea pig 0.05%, for hamster 0.04% w/v collagenase; pH 7.6) and pre-perfusion was continued for another 10 min. For rats and guinea pigs the perfusion rate was 40 ml/min, for hamsters it was 30 ml/min. The crude cell suspension was treated according to the method of Seglen (1973), and the final pellet was resuspended in a Ca2+-containing HEPES-TES buffer solution (pH 7.6) and diluted to a density of 10×10^6 cells/ml. Trypan-blue exclusion showed the presence of about 95% viable cells.

Preparation of S9 mix from isolated hepatocytes

10 ml of a suspension of 10×10^6 liver cells/ml were centrifuged at 1600 xg for 2 min. The pellet was suspended in 5 ml of HEPES-TES buffer containing 2% albumin, glucose-6-phosphate (15 umoles/ml) and NADP (12 umoles/ml). This suspension was sonicated twice for 30 sec, with an interval of 25 sec in an MSE Ultrasonic Disintegrator (100 W) at maximal energy. During this

step the suspension was cooled on ice. Next, the sonicated suspension was centrifuged at 9000 xg for 20 min. To the supernatant, 5 ml of the HEPES-TES buffer containing albumin, glucose-6-phosphate, NADP, and as indicated in the Results section, 0.2 or 2 mM acetyl-CoA were added. All manipulations were performed at 4 oC and under sterile conditions.

Salmonella/hepatocyte suspension assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile vials (inner diameter 24 mm, height 55 mm), each containing 387.5 ul of hepatocyte suspension (10 x 10^6 viable cells/ml), 0.1 ml of an overnight-grown suspension of Salmonella typhimurium (about 1.5 x 10^9 bacteria/ml) and 12.5 ul of a solution (in DMSO) of the compound under test, were incubated at 37 oC for 2 h. Next, each mixture was plated with a molten topagar containing only biotin (no histidine).
The number of revertant colonies was counted after incubation at 37 oC for 48 h.

Salmonella/disrupted hepatocyte suspension assay

In this assay, Salmonella typhimurium and the compound under test were incubated as described for the Salmonella/hepatocyte assay. However, instead of a suspension of hepatocytes, we added 387.5 ul of S9 prepared from an equal amount of the same isolated hepatocytes as used in the parallel Salmonella/hepatocyte assay. Molten topagars contained only biotin (no histidine).

Mutagenicity of benzidine and 4-aminobiphenyl

In Figure 1A the mutagenicity of benzidine is shown towards Salmonella typhimurium TA1538 using liver preparations of rat, hamster or guinea pig as the metabolic factor. The results show that in the rat and hamster the mutagenicity of benzidine was much higher with intact hepatocytes than with S9 prepared from disrupted hepatocytes. In the guinea pig liver preparations this difference was less evident. The mutagenic potency of benzidine with the rat and hamster liver preparations was relatively high. In contrast, with the guinea pig liver preparations the mutagenic potency of benzidine was almost negligible.
Figure 1B shows the mutagenicity of 4-aminobiphenyl towards Salmonella typhimurium TA1538 using intact hepatocytes and S9 prepared from disrupted hepatocytes as the metabolic factor. In contrast to benzidine, for all three species, the mutagenicity of 4-aminobiphenyl was higher when S9 was used instead of intact hepatocytes. No obvious interspecies differences in mutagenic potency of 4-aminobiphenyl were observed when liver preparations of rat, hamster and guinea pig were applied as the metabolic factor.
In the presence of rat and hamster hepatocytes or S9, 4-aminobiphenyl showed a much lower mutagenic potency than benzidine. When guinea pig S9 was used, 4-aminobiphenyl was slightly more mutagenic than benzidine.

Influence of acetyl-CoA

The effects of acetyl-CoA, the cofactor for the acetylation reaction, on the mutagenicity of benzidine and 4-aminobiphenyl are presented in Figures 1 and 2, respectively. For each of the three species, it was observed that the mutagenicity of benzidine increased when the S9 preparation was supplemented with

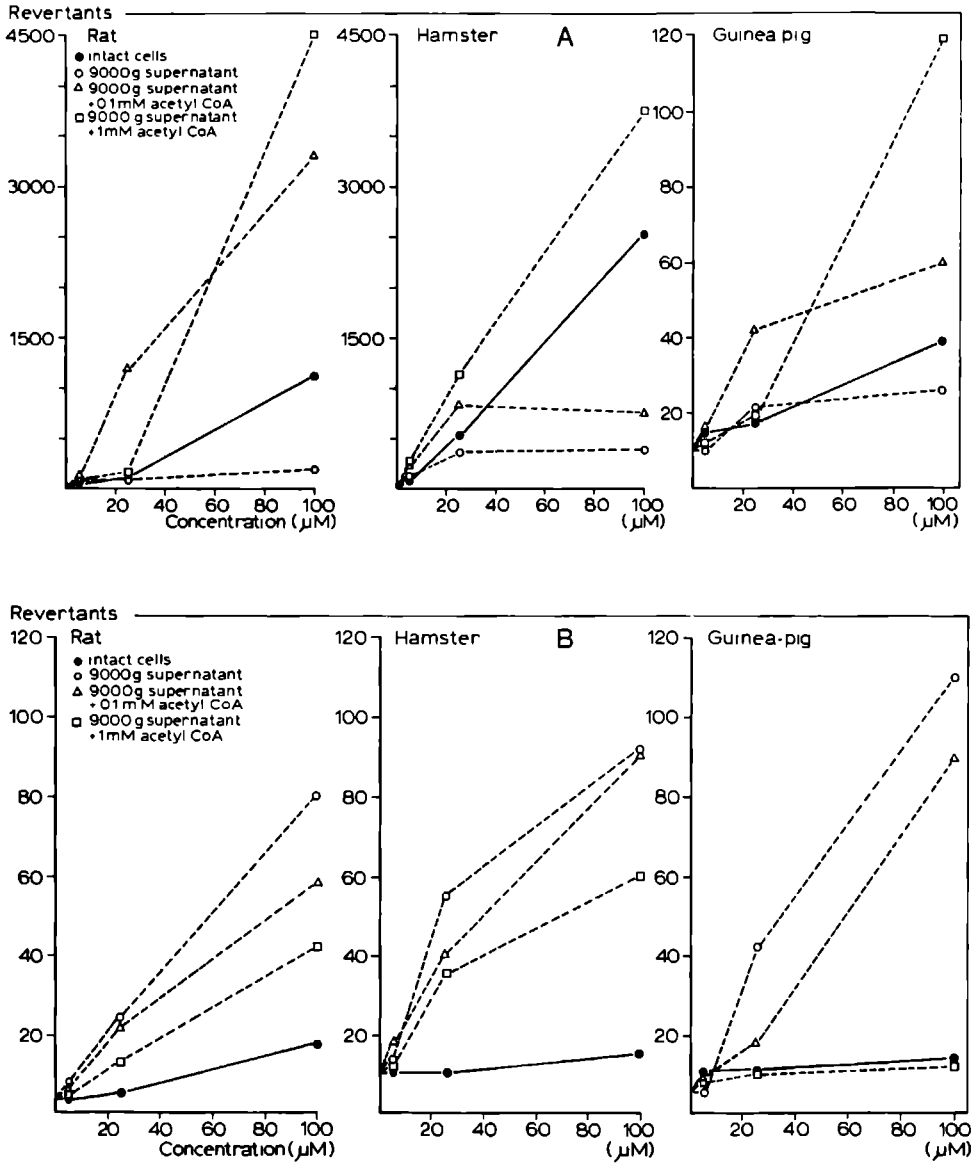

Fig. 1. Mutagenicity of benzidine (A) and 4-aminobiphenyl (B) towards Salmonella typhimurium TA1538 after metabolic activation with intact hepatocytes (●), 9000 xg supernatant prepared from disrupted hepatocytes (○), 9000 xg supernatant prepared from disrupted hepatocytes supplemented with acetyl-CoA, 0.1 mM (△) or 1 mM (□), from rat, hamster and guinea pig. Values are the means of triplicate determinations.

acetyl-CoA. This increase in mutagenicity was dependent on the concentration of acetyl-CoA. On the contrary, the mutagenicity of 4-aminobiphenyl decreased when acetyl-CoA was added to the S9. For each of the three species the mutagenicity values at 1 mM acetyl-CoA were lower than those at 0.1 mM. The decrease was most pronounced after incubation with the guinea pig S9 fraction, when the mutagenicity of 4-aminobiphenyl completely disappeared in the presence of 1 mM acetyl-CoA.

DISCUSSION

In many animal species, N-acetylation is a major pathway in the metabolism of arylamines (Irving, 1979). N-Acetylation can be followed by reactions such as N-hydroxylation and subsequent esterification, resulting in the generation of electrophilic metabolites that can react with DNA. For carcinogenic amines and their N-acetyl-derivatives, N-hydroxylation is an essential step in the metabolic activation and an absolute requirement for their carcinogenic action (Irving, 1979).

The present results demonstrate the great importance of the acetylation reaction in the biotransformation of benzidine and 4-aminobiphenyl into mutagenic products. It is interesting that for the bifunctional arylamine, benzidine, the acetylation reaction appears to increase the mutagenicity (Figure 1A) whereas for the monofunctional arylamine, 4-aminobiphenyl, the acetylation results in a reduction of mutagenic potency (Figure 1B).

The fact that acetylation is a major metabolic pathway for arylamines has led to the proposal of Morton et al. (1979) that it may precede N-hydroxylation, thus leading, in the case of benzidine, to the production of N-hydroxy-N,N'-diacetylbenzidine. These authors also showed that this intermediate can act as a good substrate for an acyltransferase enzyme, generating products highly mutagenic towards Salmonella typhimurium. Lazear et al. (1979) and Tanaka et al. (1981) showed that N-acetylbenzidine

and N,N'-diacetylbenzidine are far more mutagenic in the Ames assay than the parent compound, benzidine. Bos et al. (1984) recently showed that intact rat hepatocytes were able to convert benzidine to a great extent into its acetylated derivatives. In contrast, the S9 liver fraction could only acetylate benzidine when the cofactor for the acetylation, acetyl-CoA, was added to the incubation medium. Our observation that benzidine is more mutagenic when intact hepatocytes instead of S9 are used as metabolic factor, are in agreement with the previous findings.

When guinea pig liver preparations were used as the metabolic factor, benzidine appeared to be almost non-mutagenic (Figure 1A). This is in accordance with the finding of Morton et al. (1979), who showed that liver microsomes of rat and hamster, unlike those of guinea pig, were able to convert N,N'-diacetylbenzidine into N-hydroxy-N,N'-diacetylbenzidine. The non-mutagenicity of benzidine in the presence of guinea pig liver preparations might be due to a lack of N-hydroxylation.

McMahon et al. (1980) showed that N-hydroxy-4-aminobiphenyl is a major metabolite of 4-aminobiphenyl after incubation with 9000 xg liver supernatant of rat, hamster and guinea pig. Miller et al. (1961) reported that in rat in vivo 4-aminobiphenyl was metabolized by successive N-acetylation and N-hydroxylation to N-hydroxy-4-acetamidobiphenyl. Connor et al. (1983) recently showed that in the Ames-assay 4-aminobiphenyl was significantly more mutagenic than the acetylated derivative, 4-acetamidobiphenyl. These authors suggested that the lower mutagenicity of 4-acetamidobiphenyl, was probably due to a lower N-hydroxylation of this compound. These findings and our mutagenicity data of 4-aminobiphenyl lead to the assumption that for all three animal species in intact hepatocytes and in S9 supplemented with acetyl-CoA, 4-aminobiphenyl is converted into the less mutagenic 4-acetamidobiphenyl.

The data of Figures 1 and 2 show that especially with intact hepatocytes of rat and hamster as the metabolic factor, benzidine is much more mutagenic than 4-aminobiphenyl. This is in agreement with the findings of Bos et al. (1980) who found that the mutagenic activity in urine of rats was much higher after

treatment of these animals with benzidine than after an equal dose of 4-aminobiphenyl. These authors also showed that in an intrasanguineous host-mediated assay with rats, benzidine was more mutagenic than 4-aminobiphenyl (Bos, 1982).
Many interspecies differences in the toxic effects of arylamines are reported. The present results emphasize that isolated hepatocytes may be very helpful in the study of parameters determining such variations. This holds particularly for studies of interspecies differences in the multi-step activation of premutagens, such as certain arylamines.

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Chapter IV

METABOLIC ACTIVATION OF N-ACETYLBENZIDINE AND N,N'-DIACETYLBENZIDINE TO MUTAGENS BY ISOLATED HEPATOCYTES AND THE 9000 XG LIVER SUPERNATANT FROM RAT, HAMSTER AND GUINEA PIG

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SUMMARY

The metabolic activation of N-acetylbenzidine (MABZ) and N,N'-diacetylbenzidine (DABZ), forming products mutagenic towards Salmonella typhimurium TA1538, was studied with isolated hepatocytes from rat, hamster and guinea pig and the S9 fraction (9000 xg supernatant) prepared from these hepatocytes. Special attention was given to the influence of acetyl-CoA, the cofactor for N-acetylation, on the mutagenicity of these arylamides.
The rat and guinea pig S9 preparation activated MABZ as well as DABZ to a much higher degree than the intact hepatocytes of these animal species. Addition of acetyl-CoA to the S9 preparation decreased the mutagenicity of MABZ and DABZ.
On the contrary, for the hamster the mutagenicity of MABZ and DABZ appeared to be lower with the S9 preparation than with intact hepatocytes. Addition of acetyl-CoA to the S9, here increased the mutagenic activity of these arylamides.
In the presence of intact hepatocytes obvious interspecies differences were observed in the activation of MABZ and DABZ. DABZ was far more effectively activated by hamster hepatocytes than by rat hepatocytes. This was not found with MABZ. Both substrates were poorly activated by guinea pig hepatocytes.

INTRODUCTION

The arylamine benzidine (BZ) was epidemologically shown to be a human carcinogen, producing tumors of the urinary bladder (Zavon, 1973). Tumors of the bladder have also been observed in dogs following a long period of feeding with BZ (Spitz, 1950). In experimental animals like mice, rats and hamsters tumors of the liver have been observed.
In the last few years BZ has received an increasing amount of attention. Interest in this arylamine was partly stimulated by the observation that BZ-based azo dyes can be reduced to free BZ

by azoreductase of the intestinal flora and the liver. Azo dyes derived from BZ and its congeners are commonly used in dyeing textiles, in plastic, paper, leather and wood stains.
Recent investigations demonstrated that following the administration of BZ-based dyes to people and animals, BZ, MABZ and DABZ could be detected (Bos, 1984; Lowry, 1980; Lynn, 1980; Tanaka, 1981).
One of the major routes for the metabolism of carcinogenic arylamines in a variety of species, including man, involves N-acetylation (Bos, 1984; Haley, 1982; Lower, 1973; Morton, 1979; Troll, 1963). The enzyme responsible for this metabolic step is a acetyl-CoA-dependent N-acetyltranferase present in mammalian liver. The N-acetylated products are subject to further biotransformation like N-hydroxylation, and esterification of the N-hydroxy-moiety catalyzed by sulfotransferase (Morton, 1980) or (N,O)-acyltransferase (Morton, 1979) to produce genotoxic products. In a previous study we found that N-acetylation in rat, hamster and guinea pig hepatocytes is an important step in the metabolic activation of BZ into mutagenic products.
In the present investigation we compared the mutagenicity of MABZ and DABZ - the acetylated derivatives of BZ - in the Salmonella/hepatocyte assay using isolated hepatocytes of rat, hamster and guinea pig. Experiments with intact hepatocytes and the postmitochondrial fraction (S9) from these cells were designed to evaluate the significance of the N-acetylation reaction in the mutagenic activation of MABZ and DABZ.

MATERIALS AND METHODS

Chemicals

MABZ and DABZ were obtained from ICN Pharmaceuticals, Inc. (Plainview, NY, USA). and were purified by recrystallization (purity>97%, checked with HPLC). D-Biotin, L-histidine-HCL, collagenase type 1, nicotinamide adenine dinucleotide phosphate

(NADP) disodium salt and glucose-6-phosphate (G-6-P) dipotassium salt were obtained from Sigma (St. Louis, Missouri, USA). Acetyl-CoA was obtained from Boehringer (Mannheim, FRG). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain). All other chemicals used were of high purity.

Animals

The species of animals used in this study were male Wistar rats (app. 200 g), male albino guinea pigs (app. 400 g) and male golden Syrian hamsters (app. 90 g).

Preparation of isolated hepatocytes

The procedure was based on the methods described by Berry and Friend (1969) and by Seglen (1973), with some modifications. All manipulations were performed under sterile conditions. Before the procedure, the animals were anesthetized by i.p. injection of sodium pentobarbital (60 mg/ml distilled water); 0.2 ml for rats, 0.7 ml for guinea pigs and 0.1 ml for hamsters. After pre-perfusion (15 min for rat and guinea pig, and 20 min for hamster) of the liver with a Ca2+-free HEPES buffer (pH 7.4), this buffer was replaced by a collagenase-containing HEPES buffer (for rat and guinea pig 0.05%, for hamster 0.04% (w/v) collagenase; pH 7.6) and perfusion was continued for a further 10 min. For rats and guinea pigs the perfusion rate was 40 ml/min, for hamsters it was 30 ml/min. After incubation of the crude cell suspension in a shaking waterbath (120 rpm) at 37 oC for 30 min, filtration, gentle centrifugation and cellwashing, the final pellet was resuspended in a HEPES-TES buffer solution (pH 7.6) containing 2% albumin. This suspension was then diluted to a density of 10 x 10^6 viable cells/ml. Trypan-blue exclusion indicated the viability to be about 95%.

Preparation of S9 mix from isolated hepatocytes

10 ml of a suspension of 10 x 10^6 liver cells/ml were centrifuged at 1600 xg for 2 min. The pellet was suspended in 5 ml of HEPES-TES buffer containing 2% albumin, glucose-6-phosphate (15 umoles/ml) and NADP (12 umoles/ml). This suspension was sonicated twice for 25 sec, with an interval of 30 sec in an MSE Ultrasonic Disintegrator (100 W) at maximal energy. During this step the suspension was cooled on ice. Next, the sonicated suspension was centrifuged at 9000 xg for 20 min. To the supernatant, 5 ml of the HEPES-TES buffer containing albumin, glucose-6-phosphate, NADP, and as indicated in the Results section, 0.2 or 2 mM acetyl-CoA were added. All manipulations were performed at 4 oC and under sterile conditions.

Salmonella/hepatocyte suspension assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile scintillation vials (20 ml) each containing 387.5 ul of hepatocyte suspension (10 x 10^6 viable cells/ml), 0.1 ml of an overnight-grown suspension of Salmonella typhimurium, strain TA1538 (about 1.5 x 10^9 bact./ml) and 12.5 ul of a solution (in DMSO) of the compound under test, were incubated at 37 oC for 2 h. Next, each mixture was plated with a molten top agar containing only biotin (no histidine). The number of revertant colonies was counted after incubation at 37 oC for 48 h.

Salmonella/disrupted hepatocyte suspension assay

In this assay, Salmonella typhimurium, TA1538, and the compound under test were incubated as described for the Salmonella/hepatocyte assay. However, instead of a suspension of hepatocytes, we added 387.5 ul of S9 mix prepared from an equal amount of the same isolated hepatocytes as used in the parallel

Salmonella/hepatocyte assay.

RESULTS

Mutagenicity of MABZ and DABZ

Figure 1A shows the mutagenicity of MABZ towards Salmonella typhimurium TA1538 after metabolic activation with intact hepatocytes and S9 mix prepared from disrupted hepatocytes of rat, hamster and guinea pig. Highest values were obtained with the liver preparations of rat and hamster. These results further demonstrate that with the S9 mix of rat and guinea pig the mutagenic activation of MABZ is much higher than with intact hepatocytes of these species. In contrast, intact hamster hepatocytes seem to activate MABZ better than the S9 mix, at concentrations of MABZ exceeding 25 uM.

Figure 1B represents the mutagenicity of DABZ towards Salmonella typhimurium TA1538 after metabolic activation by the various liver preparations. As was observed for MABZ (Figure 1A), the mutagenicity of DABZ was also significantly higher with S9 mix than with intact hepatocytes of rat and guinea pig. However, with hamster hepatocytes the mutagenic activity of DABZ at high concentration appeared to be higher than with the S9 mix, whereas at low concentrations of DABZ the S9 mix was more active. Again the guinea pig liver preparations were found to be poor activators, whereas with the rat and hamster liver preparations a relatively high mutagenic activity of DABZ was obtained. However, hamster hepatocytes appeared far more effective in activating DABZ than rat hepatocytes.

In the presence of the rat hepatocytes, the mutagenic potency of MABZ was much higher than that of DABZ. However, such an obvious difference was not observed with the hamster or guinea pig hepatocytes.

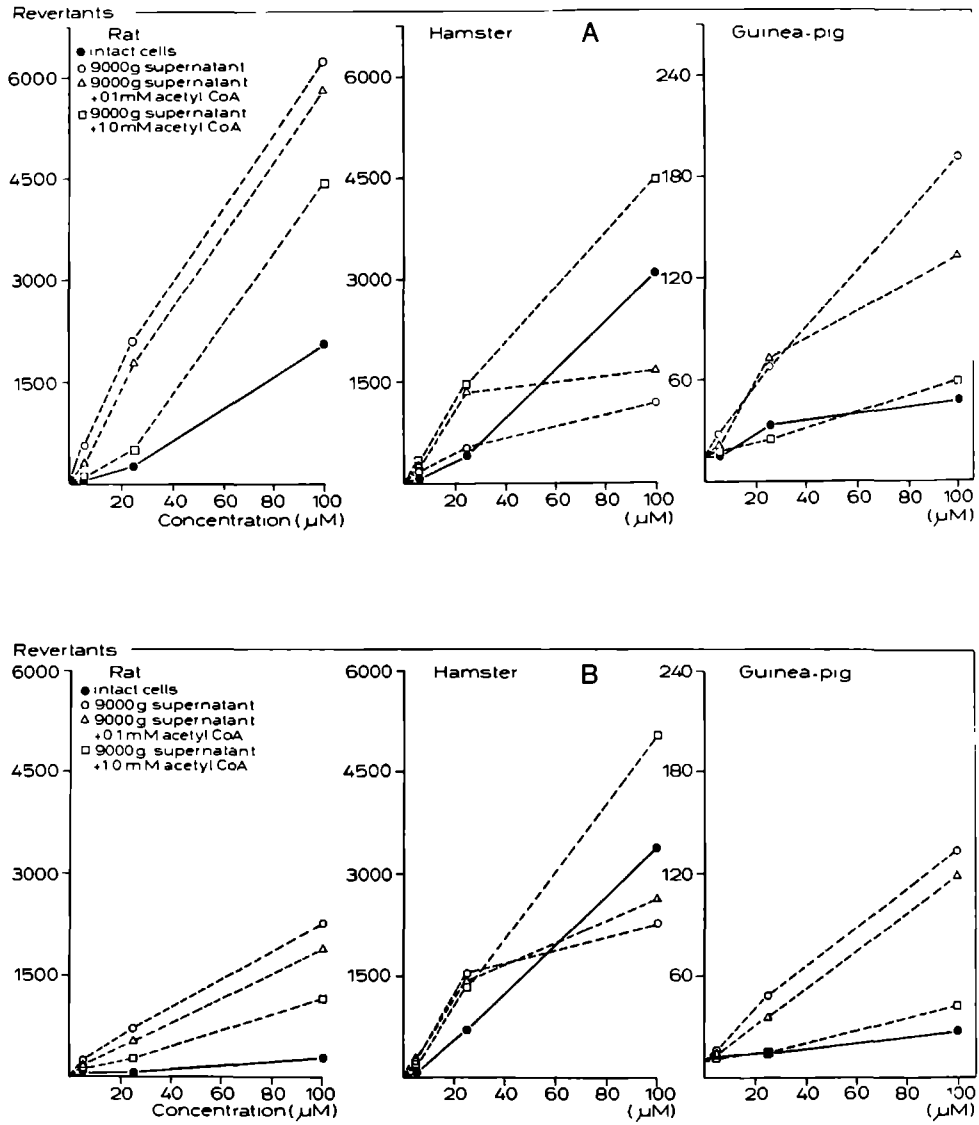

Fig. 1. Mutagenicity of MABZ (A) and DABZ (B) towards Salmonella typhimurium TA1538 after metabolic activation with intact hepatocytes (●), 9000 xg supernatant prepared from disrupted hepatocytes (○), 9000 xg supernatant prepared from disrupted hepatocytes supplemented with acetyl-CoA, 0.1 mM (△), or 1 mM (□), from rat, hamster and guinea pig. Values are the means of triplicate determinations.

Influence of acetyl-CoA

Figures 1A and 1B also show the effects of the addition of acetyl-CoA to the S9 preparation on the mutagenicity of MABZ and DABZ. For the rat and guinea pig, supplementation of acetyl-CoA - the cofactor for N-acetylation - caused a decrease in mutagenicity of MABZ as well as of DABZ. On the contrary, when acetyl-CoA was added to the hamster S9 preparation the mutagenic activity of MABZ as well as DABZ increased. The influence on mutagenicity appeared to be concentration dependent, being greater at 1 mM than at 0.1 mM acetyl-CoA.

DISCUSSION

In a previous study we demonstrated that enrichment of the S9 mix of rat, hamster and guinea pig hepatocytes with acetyl-CoA, increased the capacity of mutagenic activation of benzidine (Neis, 1984). The present results show that for the rat and guinea pig, addition of acetyl-CoA to the S9 mix decreased the mutagenicity of MABZ and DABZ. A reversed effect of acetyl-CoA was found with the hamster S9 mix (Figures 1A and 1B). Apparently, for guinea pig liver preparations, the difference between the intact cells and the S9 mix in their capacity to activate these arylamides disappears on the addition of up to 1 mM acetyl-CoA. For rat-liver preparations, this difference is reduced by the addition of acetyl-CoA (Figures 1A and 1B).
Recently, Bos et al. (1984) reported that intact rat hepatocytes were able to acetylate BZ to a great extent into MABZ and DABZ. The S9 mix could only acetylate BZ when acetyl-CoA was added to the incubation medium. We therefore suggest that in the S9 mix a low acetylating activity (due to a lack of acetyl-CoA) is overruled by a high activity of deacetylation. On the other hand, in the intact hepatocytes and in the S9 mix supplemented with acetyl-CoA, the equilibrium of the acetylation/deacetylation reaction favours acetylation. This means that after incubation

of the latter systems with MABZ or DABZ, mainly DABZ will be present.

We observed a low mutagenic activation of DABZ compared with MABZ and BZ (Neis, 1984) using intact rat hepatocytes. This is in agreement with in vivo carcinogenicity and DNA-binding studies (Martin, 1982 and 1983; Morton, 1981). Morton et al. (1981) presented carcinogenicity data showing BZ to be more potent than DABZ in the rat. Martin et al. (1982 and 1983) recently reported that administration of BZ or MABZ to rat or mice produced a single hepatic DNA adduct, which was identified as N-(deoxyguanosin-8-yl)-N'-acetylbenzidine, showing N-OH-N'-acetylbenzidine as a proximate carcinogenic species. Administration of DABZ produced only barely detectable binding. These authors suggested that DABZ was a poor substrate for N-hydroxylation in rats in vivo or possibly that it was rapidly detoxified.

Our observed high mutagenicity of MABZ and the even slightly higher activity of DABZ with hamster hepatocytes favours the idea that N-OH-N,N'-diacetylbenzidine rather than N-OH-N'-acetylbenzidine is the proximate genotoxic species in the hamster. This concept is in agreement with the finding of Morton et al. (1979) that hamster-liver preparations are far more active in N-hydroxylation of DABZ than rat or guinea pig liver preparations. These authors also showed that N-OH-N,N'-diacetylbenzidine was a good substrate for an (N,O)-acetyltransferase, generating products highly mutagenic to Salmonella typhimurium.

We observed relatively low mutagenic activities of BZ (Neis, 1984), MABZ and DABZ after metabolic activation with guinea pig hepatocytes and S9 mix. This may be due to a low N-hydroxylating capacity (Miller, 1964; Morton, 1979) or a high detoxification of formed N-hydroxy-metabolites (Razzouk, 1980) in the guinea pig. The present data, together with those obtained from studies mentioned above, can be summarized in a tentative scheme, as illustrated in Figure 2.

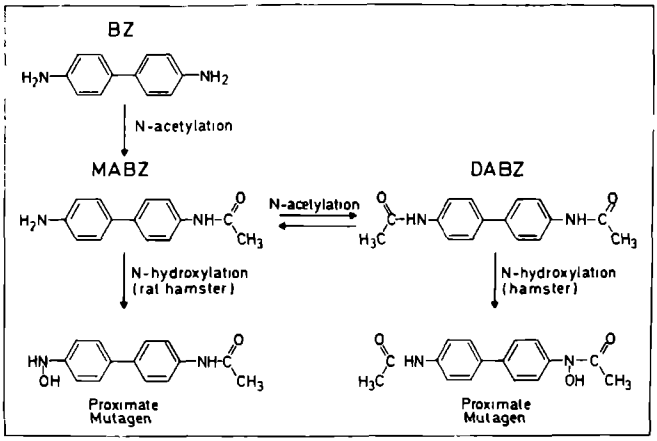

Fig. 2. Tentative scheme for the metabolic activation of MABZ and DABZ in rat and hamster.

Differences found between rat and hamster may contribute to differences in the substrate specificity of N-hydroxylating enzymes. The low capacity of mutagenic activation of the guinea pig is probably caused by poor N-hydroxylation of both MABZ and DABZ, or to an effective detoxification.

Because of the importance of the N-acetylation in the metabolic activation of arylamines, we suggest that intact hepatocytes are probably a better tool in the screening of arylamine genotoxicity than subcellular liver fractions, like the commonly used S9 mix.

ACKNOWLEDGEMENTS

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BENZIDINE, N-ACETYLBENZIDINE AND N,N'-DIACETYLBENZIDINE EVOKED DNA-EXCISION REPAIR IN ISOLATED HEPATOCYTES OF RAT, HAMSTER AND GUINEA PIG

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SUMMARY

DNA-excision repair in isolated hepatocytes of rat, hamster and guinea pig, as a result of exposure to benzidine (BZ), N-acetylbenzidine (MABZ) and N,N'-diacetylbenzidine (DABZ), was measured as 3H-Thymidine (3H-TdR) incorporation into the hepatocellular DNA.
With the hepatocytes of all three animal species a dose-dependent DNA-repair was found after exposure of these cells to all three compounds. However, obvious interspecies differences were observed in the extent of the induced DNA-repair. BZ, MABZ and DABZ were more effective in inducing a repair-response in hamster hepatocytes than in rat hepatocytes. All three substrates induced only a slight increase in DNA-repair in the guinea pig hepatocytes.
BZ, MABZ and DABZ appeared to be equal active in inducing DNA-repair in the quinea pig hepatocytes. However, in the rat and hamster hepatocytes MABZ and DABZ induced a far more effective DNA-repair than BZ. The results indicate, that the N-acetylation plays an important role in the metabolic activation of BZ to DNA-damaging products in the rat and hamster liver.

INTRODUCTION

The arylamine BZ has been epidemiologically shown to produce tumors of the urinary bladder in humans (Zavon, 1973). In dogs also tumors of the bladder have been observed after feeding of BZ. In rodent species like rat, hamster and mouse, tumors of the liver have been observed.
The carcinogenic activity of many precarcinogens is believed to result mainly from the interaction of the ultimate carcinogenic metabolites with the DNA of the target cells. Therefore measurement of alterations in the DNA after exposure to these compounds may be a suitable tool for detecting their potential

genotoxicity. The measurement of DNA-excision repair in hepatocytes as unscheduled DNA-synthesis (UDS) following chemical induced DNA-damage has been shown to be a valuable tool in assessing carcinogenic properties of chemicals (Brouns, 1979; McQueen, 1981; Mitchell, 1983; Olson, 1983; Probst, 1981; Williams, 1982).

We recently showed that BZ, MABZ and DABZ are metabolized by isolated hepatocytes of rat, hamster and man to products highly mutagenic towards Salmonella typhimurium TA1538 (Neis, 1984, 1985a and 1985b). However, all three compounds were poorly activated by guinea pig hepatocytes (Neis, 1984 and 1985b). In this Salmonella/hepatocyte assay, bacterial mutagenicity can be used as a parameter of extra-hepatocellular appearance of proximate or ultimate mutagenic metabolites. Whereas, hepatocellular DNA-excision repair can be used as endpoint for genotoxic effects within the hepatocytes.

In the present study we have examined the capability of suspensions of freshly isolated hepatocytes of rat, hamster and guinea pig, to metabolize BZ, MABZ and DABZ to DNA-damaging products by measurement of DNA-excision repair, estimated via the incorporation of 3H-TdR into the hepatocellular DNA with liquid-scintillation counting.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following companies: BZ was from Merck (Darmstadt, FRG); MABZ and DABZ were obtained from ICN Pharmaceutical Inc. (Plainview, NY, USA). MABZ was purified by recrystallization from ethanol. DABZ contained 18% MABZ and was purified as follows: the DABZ sample was dissolved in 1N HCl and the DABZ was extracted with ethylacetate and evaporated to dryness. This preparation was further purified by recrystallization from ethanol. (Purity: MABZ>97%, DABZ>99%,

checked with HPLC). Collagenase (from Clostridium histolyticum) was obtained from Boehringer (Mannheim, FRG); Eagle's minimal essential medium including Hank's salts, and foetal calf serum were from biomed (Gibco); 3H-Thymidine (3H-TdR), spec. act. 26 Ci/mmol was from the Radiochemical Centre (Amersham, UK). All other chemicals were of high purity.

Preparation of isolated hepatocytes

Male Wistar rats (app. 200 gr), male albino guinea pigs (app. 400 gr) and male golden Syrian hamsters (app. 100 gr) were used for the preparation of isolated hepatocytes. The hepatocytes were isolated by a collagenase perfusion technique as previously described (Neis, 1984 and 1985b). All manipulations were carried out under sterile conditions.

DNA-repair assay

Hepatocellular DNA-excision repair was measured as 3H-TdR incorporation into DNA by a rapid and simple technique via liquid scintillation counting as described by Brouns et al. (1979), with slight modifications. The isolated hepatocytes were resuspended in Eagle's minimal essential medium, supplemented with Hank's salts, 10% foetal calf serum, streptomycin 0.01% and penicillin-G 100 U/ml. In a shaking waterbath (120 rpm), closed scintillation vials (20 ml) each containing 487.5 ul of hepatocyte suspension (1×10^6 viable cells/ml) and 12.5 ul of a solution (in DMSO) of the compound under test, were incubated in triplicate at 37 oC for 1 h. To three vials, serving as control, 12.5 ul DMSO was added. After incubation, 3H-TdR was added to each vial to a final concentration of 1 uCi/ml, whereafter incubation was continued for 2 h. Next, 2 ml of saline was added to each vial. Each mixture was transferred to centrifuge tubes and centrifuged at 3000 xg for 10 min, and the supernatants were carefully decanted. To the cell pellets, 1 ml 6 M NaSCN solution was added

under vigorous shaking, and were left to stand overnight at room temperature to achieve complete lysis of the cells. Subsequently, after addition of 1 ml carrier DNA solution (0.1 mg DNA/ml), macromolecules were precipitated by addition of 2 ml 20% ice-cold TCA solution under vigorous shaking. After standing at -20 oC for 30 min the tubes were centrifuged at 3000 xg for 15 min. The pellet was washed once with 70% ethanol and once with cold 0.5N PCA, after which the precipitated DNA was selectively solubilized with 0.5 ml 0.5N PCA at 80 oC for 60 min. The tubes were then centrifuged at 3000 xg for 15 min, and the supernatant added to scintillation vials. After addition of 5 ml Aqua Luma to each vial, the 3H-radioactivity was measured by a Philips liquid-scintillation analyzer (efficiency 39-42%).

RESULTS

BZ, MABZ and DABZ evoked DNA-repair

Benzidine and its acetylated derivatives evoked UDS in the isolated hepatocytes of rat, hamster and guinea pig. The 3H-Tdr incorporation was found to be concentration dependent (Figure 1). Almost in all cases, beyond a certain concentration of test compound an abrupt decline in incorporation of 3H-TdR was observed, possibly due to injurious or inhibitory effects on the processes involved in the DNA-repair, since the cellular viability (as judged by trypan-blue exclusion) was almost uneffected at the concentrations used.

Rat

From the concentration-effect curves it can be derived that in the hepatocytes of rat an obvious increase in DNA-repair synthesis occurred when exposed to BZ, MABZ and DABZ. The acetylated derivatives, e.g. MABZ and DABZ, appeared to induce a higher DNA-repair than the parent compound BZ.

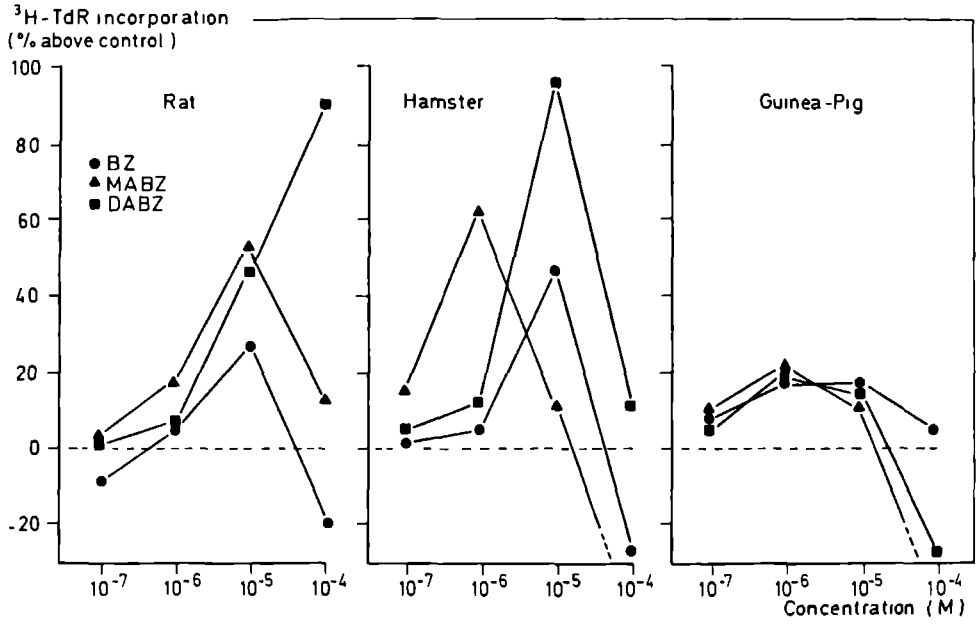

Fig. 1. Incorporation of 3H-TdR into the DNA of isolated hepatocytes of rat, hamster and guinea pig as result of exposure to different concentrations of BZ(●), MABZ(▲) and DABZ(■). Values are the means (SEM-values less than 10% of the mean) of three separate experiments with hepatocyte preparations of different animals, each assayed in triplicate.

Hamster

The levels of DNA-repair evoked in hamster hepatocytes were about the same as those in the rat hepatocytes, however for MABZ and DABZ the maximum increase in 3H-Tdr incorporation was reached at concentrations 10 times lower than with the rat hepatocytes. In the hamster hepatocytes, like in the rat hepatocytes, MABZ and DABZ appeared more effective than BZ.

Guinea pig

All three compounds appeared to evoke only a slight increase in repair in the guinea pig hepatocytes. This low increase in DNA-repair is probably not attributed to a low activity of the DNA-repair system of these cells, because in reference experiments we found a relatively active DNA-repair, when exposed to 4-nitroquinoline-1-oxide, a well known DNA damaging agent. With the guinea pig hepatocytes, in contrast to the rat and hamster hepatocytes, almost similar concentration-effect curves were obtained with all three compounds.

DISCUSSION

Many investigations have shown that DNA-excision repair represents a reliable indicator of prior DNA-damage caused by chemical carcinogens. The present data show that in rat, hamster and guinea pig hepatocytes, repair synthesis is evoked in response to treatment with BZ, MABZ and DABZ, demonstrating the capability of these cells to metabolize these compounds to DNA-damaging products (Figure 1).

In a previous study (Neis, 1984) we found with rat, hamster and guinea pig hepatocytes, that the N-acetylation is an important step in the metabolic activation of BZ to products mutagenic to Salmonella typhimurium. The present results show that in rat and hamster hepatocytes, the acetylated BZ-derivatives MABZ and DABZ are more effective than BZ itself (Figure 1). This supports the concept that N-acetylation is a very important step in the metabolic activation of BZ to genotoxic products in rat and hamster liver. Martin et al. (1980) have also reported the importance of the N-acetylation reaction in BZ genotoxicity. These authors showed that in the liver of rats or mice exposed to BZ only one major DNA-adduct was obtained namely N-(deoxyguanosin-8-yl)-N'-acetylbenzidine. They suggested from these findings that the most likely proximate carcinogenic

metabolite of BZ is N-OH-N'-MABZ.

A more effective DNA-repair, as response to exposure to BZ, MABZ and DABZ, was obtained in hamster than in rat hepatocytes (Figure 1). Kornbrust et al. (1984) recently also showed that BZ induced a higher DNA-repair in primary cultures of hamster hepatocytes than rat hepatocytes. They also reported that the direct alkylating agent methylmethanesulfonate (MMS) induced a similar DNA-repair response in rat and hamster hepatocytes. Therefore, it is more likely to attribute the observed greater DNA-repair response in the hamster hepatocytes after exposure to BZ, MABZ and DABZ (Figure 1), to a higher activating potential of the hamster hepatocytes than to differences in DNA-repair capacity between the rat and hamster hepatocytes. The greater DNA-repair response to BZ, MABZ and DABZ, in hamster hepatocytes in comparison with rat hepatocytes is consistent with our previous observation that the mutagenic activity of these compounds to Salmonella typhimurium was higher in the presence of hamster than rat hepatocytes (Neis, 1984 and 1985b). These species differences are not surprising since it is known that the hamster liver posesses a far more effective N-acetylase, N-hydroxylase, N,O-acyltransferase and sulfotransferase activity (important steps in the metabolic activation of arylamines and arylamides to genotoxic products) than the rat liver (Kato, 1983; Morton, 1979 and 1980). Contrarily, Kennely et al. (1984) reported a two-fold higher DNA-adduct formation by MABZ in the rat than in the hamster in the liver in-vivo, as well as in liver slices in-vitro.

Recently, we reported a relative low mutagenic activity of DABZ (compared to BZ and MABZ) towards Salmonella typhimurium TA1538 using intact isolated rat hepatocytes as metabolizing system (Neis, 1985b). The present results however, show DABZ highly active in inducing DNA-repair synthesis in rat hepatocytes (Figure 1). In the Salmonella/hepatocyte assay, however, bacterial mutagenicity represents extra-hepatocellular appearance of proximate and ultimate mutagenic metabolites, whereas in the hepatocyte/repair assay, DNA-excision repair is an endpoint of genotoxic effects within the hepatocytes. The close proximity of

metabolic activation and the target hepatocellular DNA in the hepatocyte/repair assay, can be expected to increase sensitivity to those compounds, whose reactive intermediates are produced in relative low concentration and/or have very short half-lives. This may explain the relative higher genotoxicity of DABZ in the DNA-repair assay than in the Salmonella/hepatocyte assay.
The relative small increase in DNA-repair in the guinea pig hepatocytes when exposed to BZ, MABZ and DABZ is consistent with our previous observed low mutagenicity of these compounds when activated by guinea pig hepatocytes (Neis, 1984 and 1985b). It is also consistent with the reported resistance of the quinea pig to the carcinogenic action of arylamines and arylamides, e.g. 2-aminofluorene and 2-acetylaminofluorene. The observed low genotoxicity may be due to a low rate of N-hydroxylation (Morton, 1979; Miller, 1964) or a high detoxification of formed N-hydroxy-metabolites (Razzouk, 1980) in the guinea pig hepatocytes.
The results of this study again underline that differences in biotransformation contribute to interspecies variations in susceptibility to chemical carcinogens.

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Chapter VI

MUTAGENICITY OF ARYLAMINES AND ARYLAMIDES AFTER METABOLIC ACTIVATION WITH ISOLATED DOG AND HUMAN HEPATOCYTES

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SUMMARY

The mutagenicity of benzidine (BZ), N-acetylbenzidine (MABZ), N,N'-diacetylbenzidine (DABZ), 4-aminobiphenyl (4-AB) and 2-aminoanthracene (2-AA) towards Salmonella typhimurium strain TA1538 was measured in the presence of isolated hepatocytes from dog and man. The influence of paraoxon, an inhibitor of the deacetylation reaction, on the mutagenicity of these compounds was also investigated.
Obvious interspecies differences in the mutagenic activation of benzidine and its acetylated-derivatives were seen. However, with liver cell preparations from both species it was found that MABZ and DABZ were more mutagenic than BZ itself. 4-AB appeared to be weakly mutagenic in the presence of human hepatocytes but non-mutagenic with dog hepatocytes. 2-AA was highly mutagenic in both species. When human hepatocytes were used as the metabolic factor, the mutagenicity of all arylamines decreased in the presence of paraoxon. With dog hepatocytes, however, the mutagenicity of all arylamines except DABZ was enhanced in the presence of paraoxon.

INTRODUCTION

Since the development of a method for in situ perfusion of the liver with collagenase, isolated hepatocytes have been used extensively to study the biotransformation and genotoxicity of xenobiotic compounds. Great differences in metabolic activation of arylamines between animal species, are held responsible for the variations in susceptibility to these compounds (Irving, 1979). Consequently, predictions about toxicity of these compounds to humans, based on animal research data are questionable.
Carcinogenic arylamines are acetylated by most animal species. The acetylated-derivatives are subject to further

biotransformation like N-hydroxylation, (N,O)-acyltransfer or sulfotransfer to produce reactive electrophiles capable of binding covalently with cellular macromolecules (Irving, 1979). Marked interspecies differences in the ability of the liver to act in the N-acetylation reaction of arylamines have been reported (Irving, 1979; Lower, 1973; Morton, 1979). Differences in N-acetylation are considered to be factors, capable of modulating susceptibility to carcinogenic arylamines (Irving, 1979; Neis, 1984). Previous investigations demonstrated the great importance of the acetylation reaction in the biotransformation of arylamines into mutagenic products (Kennely, 1984; Neis, 1984). It is known that most dogs have only a limited ability to acetylate arylamines (Bradshaw, 1955; Haley, 1982; Irving, 1979; Lower, 1973; Poirier, 1963; Scianni, 1958) in contrast to man (Haley, 1982; Scianni, 1961; Troll, 1963). In the present study we compared the mutagenicity of five structurally related arylamines in the Salmonella/hepatocyte assay using isolated dog and human hepatocytes. Special attention was given to the role of the deacetylation reaction in the metabolic activation of these compounds.

MATERIALS AND METHODS

Chemicals

BZ was purchased from Merck (Darmstadt, FRG). MABZ and DABZ were obtained from ICN Pharmaceuticals, Inc. (Plainview, NY, USA). MABZ and DABZ were purified by recrystallization (purity>97%, checked with HPLC). 4-AB and 2-AA and diethyl-p-nitrophenylphosphate (paraoxon) were from Aldrich Europe (Beerse, Belgium). D-Biotin, L-histidine-HCl, collagenase type 1, nicotinamide adenine dinucleotide phosphate disodium salt and glucose-6-phosphate dipotassium salt were obtained from

Sigma (St. Louis, Missouri, USA). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, UK). All other chemicals used were of high purity.

Preparation of isolated hepatocytes

The in situ two-step collagenase-perfusion technique described for the isolation of hepatocytes from rodent liver (Neis, 1984) was adapted into a procedure applicable for the isolation of dog and human hepatocytes. All manipulations were performed under sterile conditions.

Dog hepatocytes

Male beagle dogs, about 30 weeks old, were anesthetized with pentobarbital sodium (30 mg/kg) and heparinized. A branch of the portal vein leading to one of the minor lobes was cannulated. The liver lobe was pre-perfused in situ with approximately 300 ml saline. Then the total liver was removed from the body and transferred to a perfusion apparatus. Approximately 1 liter of a Ca2+-free HEPES buffer (pH 7.4) was pumped through the liver lobe at a rate of 50 ml/min, followed by 100 ml HEPES buffer (pH 7.6) containing 0.05% w/v collagenase. At this stage the liver lobe was cut loose from the rest of the liver and perfusion was continued with 100 ml HEPES buffer (pH 7.6) containing 0.1% w/v collagenase with recirculation for the period of 10 min. After incubation of the crude cell suspension in a shaking waterbath (120 rpm) at 37 oC for 30 min, filtration, gentle centrifugation and cellwashing, the final pellet was resuspended in a HEPES-TES buffer solution (pH 7.6) containing 2% albumin. The suspension was diluted to a density of 10×10^6 viable cells/ml. Viability was about 95% as measured by trypan-blue exclusion.

Human hepatocytes

The human normal liver sample was obtained from a 35 year old male kidney donor 3 h after he was killed in a traffic accident. The liver sample (a part of the left lobe, weighing app. 400 g) was surrounded by the hepatic capsule on all sides except for one cut surface. PVC-tubing (non-toxic quality) was inserted in a large vein on the cut surface and held in place with a ligature. Then approximately 1 liter Ca2+-free HEPES buffer (pH 7.4) was pumped through the liver at a rate of 50 ml/min. As soon as the perfusion was started it was observed that a great part of the liver sample began to blanch. After the pre-perfusion the liver was perfused successively with 200 ml of a HEPES buffer (pH 7.6) containing 0.05% w/v collagenase without recirculation, and with 100 ml HEPES buffer (pH 7.6) containing 0.1% w/v collagenase with recirculation for a period of 10 min. The total yield of isolated hepatocytes was 1.2×10^9 cells and viability was 92% as measured by trypan-blue exclusion.

Salmonella/hepatocyte suspension assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile scintillation vials (20 ml), each containing 387.5 ul of hepatocyte suspension (10×10^6 viable cells/ml), 0.1 ml of an exponential-growing culture of Salmonella typhimurium (about 2.0×10^9 bacteria/ml) and 12.5 ul of a solution (in DMSO) of the compound under test, were incubated in triplicate at 37 oC for 2 h. Next, each mixture was plated with a molten top agar containing only biotin (no histidine). The number of revertant colonies was counted after incubation at 37 oC for 48 h.

Analytical methods

The cytochrome-P450 content of the isolated hepatocytes was measured with the cytochrome-P450 CO-difference spectra after reduction with sodium dithionite according to the method of Omura and Sato (1964). Prior to the measurement of the cytochrome-P450, the hepatocyte suspension was sonicated twice for 20 sec with an interval of 30 sec in an MSE Ultrasonic Disintegrator (100W) at maximal energy. During this step the suspension was cooled on ice.
Cellular protein content was assayed by the Coomassie Brilliant Blue method , using 0.1N NaOH to lyse the cells (Godstadt, 1982). The cells were previously washed with saline to remove contaminants (e.g. albumin) in the suspension buffer. Bovine serum albumin was used as a standard.

RESULTS

Cell size, protein- and cytochrome P-450 content

For both species cell size, protein- and cytochrome-P 450 content of the isolated hepatocytes were determined (Table I).
The data in Table I clearly show that the human hepatocytes contain less protein and cytochrome P-450 than the dog hepatocytes.

Mutagenicity of BZ, MABZ, DABZ, 4-AB and 2-AA

Figure 1 shows the mutagenicity of BZ, MABZ, DABZ, 4-AB and 2-AA towards Salmonella typhimurium strain TA1538 after metabolic activation with isolated hepatocytes from dog and man.
Great interspecies differences in the mutagenic potency of BZ, MABZ and DABZ were seen. These three compounds revealed a relatively high mutagenicity after activation with human

TABLE I

CELL SIZE, PROTEIN AND CYTOCHROME P-450 CONTENT OF ISOLATED DOG AND HUMAN HEPATOCYTES

	Cell diameter(um)[a]		ug protein/ 10^6 cells[b]	pmol cytochrome P-450/ 10^6 cells[b]
	Mean	Range		
Dog	18.3	17-21	960 ± 23	210 ± 10
Man	17.7	16-19	500 ± 16	94 ± 2

a) Values obtained by microscopic measurement using a calibrated grid.

b) Values are the means (± S.E.M.) of determinations in triplicate.

TABLE II

INFLUENCE OF PARAOXON ON THE MUTAGENICITY OF BZ, MABZ, DABZ, 4-AB AND 2-AA AFTER METABOLIC ACTIVATION WITH ISOLATED DOG AND HUMAN HEPATOCYTES

	Human hepatocytes		Dog hepatocytes	
	10^{-5}M paraoxon	10^{-4}M paraoxon	10^{-5}M paraoxon	10^{-4}M paraoxon
BZ (10^{-4}M)	56	41	133	146
MABZ (10^{-4}M)	40	16	133	176
DABZ (10^{-4}M)	11	5	18	21
4-AB (10^{-4}M)	40	35	117	248
2-AA (10^{-4}M)	98	88	106	148

Values representing relative mutagenicity (% of control) are the means of determinations in triplicate.

hepatocytes, whereas with dog hepatocytes BZ was non-mutagenic, MABZ and DABZ were only weakly mutagenic. With dog hepatocytes DABZ showed a higher mutagenic activity than MABZ, whereas with the human cells MABZ was slightly more mutagenic than DABZ. However, in both cases MABZ and DABZ were more mutagenic than BZ itself.

4-AB showed a low mutagenic activity with human hepatocytes but was non-mutagenic with dog hepatocytes. No obvious interspecies differences in the mutagenic potency of 2-AA were observed. It appeared to be highly mutagenic in the presence of hepatocytes from either species.

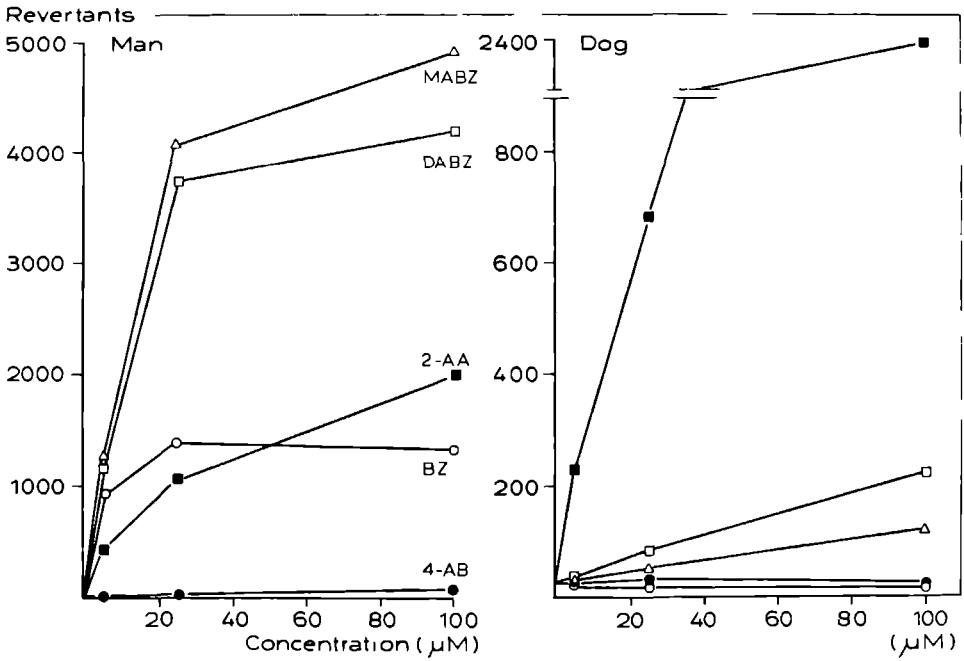

Fig. 1. Mutagenicity of BZ(O), MABZ(Δ), DABZ(□), 4-AB(●) and 2-AA(■) towards Salmonella typhimurium TA1538 after metabolic activation with intact hepatocytes from man and dog. Values are the means of determinations in triplicate.

Influence of paraoxon

The effects of paraoxon - an inhibitor of the deacetylation reaction- on the mutagenicity of the arylamines, in the presence of human and dog hepatocytes, are shown in Table II.
With human hepatocytes the mutagenicity of all arylamines was decreased in the presence of paraoxon. The decrease in mutagenicity was higher with 10 M paraoxon than with 10 M. This inhibitory effect on the mutagenicity of BZ and its acetylated-derivatives was in the order DABZ > MABZ > BZ.
The mutagenic activity of 2-AA was only weakly influenced.
On the contrary, with dog hepatocytes the mutagenicity of all arylamines except DABZ was enhanced in the presence of paraoxon. It should be noted, that the addition of paraoxon had no deleterious effect on the bacteria, as was concluded from the absence of any influence on bacterial growth. It was also ensured that hepatocellular viability was not influenced by the presence of paraoxon in these concentrations. An inhibitory effect on microsomal oxidizing capacity at the concentration used was also virtually absent.

DISCUSSION

Dog and human hepatocytes were isolated, in a viable state, using a modification of the collagenase perfusion technique, generally employed for the isolation of rodent hepatocytes. The mutagenic activation of the tested arylamines proceeded more effectively in the presence of human hepatocytes than with dog hepatocytes, although the cells of the dog contained more cytochrome-P 450. For most species, including man, it seems that the rate of N-acetylation is greater than the deacetylation rate (Haley, 1982; Irving, 1979; Lower, 1973; Morton, 1979; Scianni, 1961; Troll, 1963). However, in the dog, a species being deficient in N-acetyltransferase activity (Bradshaw, 1955; Clayson, 1959; Haley, 1982; Irving, 1979; Lower, 1973; Poirier, 1963; Scianni,

1958) but having a high deacetylation capacity towards acetylated arylamines (Clayson, 1959), deacetylation predominates. This phenomenon may partly account for the observed non-mutagenicity of BZ and the low mutagenicity of MABZ and DABZ in the presence of dog hepatocytes, and the relatively high mutagenicity of all three compounds with human hepatocytes. When in the dog hepatocytes the deacetylation reaction is inhibited by paraoxon, the mutagenicity of all arylamines except DABZ increased (Table II). This may indicate that dog hepatocytes are able to act in the N-acetylation of arylamines but that due to a high deacetylation rate the nett production of N-acetylated metabolites is negligible. The substantial decrease in mutagenicity of DABZ due to paraoxon in both species, indicates that deacetylation is an essential step in the mutagenic activation of this diacetylated derivative. On the other hand, in human hepatocytes paraoxon decreased the mutagenicity of the tested arylamines. This indicates that in this species apart from acetylation also deacetylation is involved in the generation of mutagenic products. Brouns et al. (1982) already presented data which were in favour of the role of the N-acetylation and deacetylation in the metabolic activation of BZ into mutagenic products in isolated rat hepatocytes. From the present data together with those obtained from studies mentioned above, we suggest that factors affecting the balance between acetylation and deacetylation may play an essential role in determining tissue and species susceptibility to arylamine carcinogenesis.
BZ was highly mutagenic and 4-AB was weakly mutagenic after activation with human hepatocytes, whereas both were non-mutagenic with dog hepatocytes (Figure 1). These observations are well compatible with the report that BZ and 4-AB are known potent human urinary bladder carcinogens in man (Case, 1954; Clayson, 1976; Scott, 1952; Zavon, 1973), whereas they are at best weak urinary bladder carcinogens in the dog (Deichmann, 1965; Spitz, 1950). The observed mutagenicity of BZ and 4-AB after metabolic activation with human hepatocytes indicates that the liver may contribute to the generation of carcinogenic metabolites for bladder cancer initiation in man. Phillipson et

al. (1983) recently showed that BZ was almost non-mutagenic towards Salmonella typhimurium TA1538 after metabolic activation with a human liver homogenate preparation (S9-mix) in the Ames-assay. On the contrary, using intact human hepatocytes we found BZ highly mutagenic. These findings again emphasize the usefulness of intact hepatocytes instead of subcellular liver fractions in studies of mutagenic activation. It is generally believed that the existence of interspecies differences in metabolic activation of genotoxic agents is the main problem in extrapolating animal research data to humans. Comparative studies with isolated hepatocytes from different species, including man, may be most helpful making these problems more comprehensible.

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ACTIVATION OF MUTAGENS BY HEPATOCYTES AND THE 9000 XG LIVER SUPERNATANT FROM HUMAN ORIGIN IN THE SALMONELLA TYPHIMURIUM MUTAGENICITY ASSAY
COMPARISON WITH RAT LIVER PREPARATIONS

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The mutagenicity of 10 known genotoxic compounds, of several chemical classes, was measured in Salmonella typhimurium mutagenicity assays comprising isolated human hepatocytes or human 9000 xg liver supernatant (S9) from four different individuals, as activating system.

The mutagenic activity of several compounds as determined with the Salmonella/hepatocyte suspension assay showed obvious differences as compared to the values obtained in the Salmonell/S9 plate assay. For instance, the mutagenic activity of benzidine (BZ), dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) appeared to be much higher in the hepatocyte assay than in the S9 assay. However, 2-aminofluorene (2-AF) and 2-acetylaminofluorene (2-AAF) were activated more effectively to mutagens in the S9 assay than in the hepatocyte assay. 2-AF was slightly more mutagenic than 2-AAF in the hepatocyte assay, whereas it was far more mutagenic than 2-AAF in the S9 assay. DMN was found more mutagenic than DEN in the hepatocyte assay, whereas in the S9 assay DEN appeared to be slightly more mutagenic. Furthermore, great interindividual differences in the metabolic activation of certain compounds, e.g. BZ and DMN, were observed in the hepatocyte suspension assay, whereas these variations were less evident in the S9 plate assay.

Comparison of the mutagenicity data obtained with the human liver preparations, with those obtained with rat liver preparations, showed great interspecies differences in the capacity to activate certain chemicals to mutagens.

The use of human liver preparations, in particular isolated human hepatocytes, may be of great value in studies on inter- and intraspecies variations in metabolic activation of genotoxic agents.

After Ames et al. (1975) had shown that the Salmonella/microsome assay was capable of identifying a large proportion of carcinogens, this test system has become a well developed and established in vitro mutagenicity assay, which is now widely used in laboratories throughout the world. In this short-term test a 9000 xg supernatant fraction (S9) from rat liver is applied to mediate metabolism of the test chemicals to mutagenic products. Several studies (Bartsch, 1983; Bigger, 1980; Bos, 1983; Greim, 1981; Langenbach, 1983; Neis, 1984; Jones, 1983) however, have indicated that S9 liver preparations may not properly represent the in vivo activation and deactivation processes, whereas preparations of intact hepatocytes are a better substitute in this respect.

It has also become evident that marked differences exist between species, strains and sexes in the metabolism and susceptibility to carcinogens (Kato, 1979; Motulsky, 1982; Streissinger, 1983; Williams, 1978)

In regard of the estimation of genotoxic risks to humans, the interpretation of data obtained from current in vitro mutagenicity assays (using rodent S9 fractions) is not only hampered by a difference between in vitro and in vivo conditions but also by interspecies variations.

We have recently described an isolation technique to obtain hepatocytes from rodent species (Neis, 1984 and 1985b). By modifying this technique into a procedure for isolation of hepatocytes from larger species including man, human hepatocytes could be isolated in a viable state (Neis, 1985a). The isolated human hepatocytes appeared to activate certain arylamines and arylamides effectively to products mutagenic to Salmonella typhimurium (Neis, 1985a). Obvious interspecies differences were observed in the mutagenic activation of these compounds between rodent, dog and human hepatocytes (Neis, 1984, 1985a and 1985b).

In the present study the mutagenicity of 10 known pregenotoxic agents, including some polycyclic arylhydrocarbons,

N-nitrosamines and arylamines (compounds known to be activated/deactivated via different pathways), were studied in the Salmonella mutagenicity assay using intact human hepatocytes and human liver S9 preparations as metabolizing system. The obtained results were compared with results previously obtained with rat liver preparations.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: BZ from Merck (Darmstadt, FRG); MABZ and DABZ were from ICN Pharmaceuticals, Inc. (Plainview, NY, USA) and were purified by recryatallization (purity>97%, checked with HPLC); 2-AF, 2-AAF, DMN and B(a)P from Aldrich Europe (Beerse, Belgium); DEN from Aldrich Chemical Co. Inc. (Milwaukee, USA); CP and HEH from Asta (Brackwede, FRG); 7,12-DMBA from Eastman Kodak Company; 3-MC from Koch-Light Laboratories Ltd. Collagenase (from Clostridium histolyticum) was obtained from Boehringer (Manheim, FRG) Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain). All other chemicals used were of high purity.

Subjects

In Table I data about age and sex of the four subjects are shown. Subject 1, was known to be a heavy drinker. The liver histology of this person showed some fibrosis with fatty changes and inflammatory cell infiltrates consistent with an alcoholic liver disease.

Preparation of isolated hepatocytes

Postmortal human liver tissues were obtained from kidney donors and human hepatocytes were isolated by a two-step perfusion technique, modified from that described for the isolation of rodent hepatocytes (Neis, 1984 and 1985b). All manipulations were performed under sterile conditions.

After removal from the body, the liver samples were perfused by syringe with cold saline to remove most of the blood, and subsequently placed in cold saline. Then the liver sample was kept on ice until the isolation procedure could be started (approximately 2-3 hours).

The liver sample (a part of the left lobe, about 250-400 g) was surrounded by the hepatic capsule on all sides except for one cut surface. PVC-tubings (non-toxic quality) were inserted in large veins on the cut surface and held in place with ligatures. Then approximately 2 liters of Ca2+-free HEPES buffer (pH 7.4), containing 0.5 mM EGTA, was pumped through the liver sample at a total rate of 150-200 ml/min (50 ml/min per tubing). As soon as the perfusion was started it was observed that a great part of the liver sample began to blanch. After the pre-perfusion, the liver sample was perfused successively with 200 ml of a HEPES buffer (pH 7.6), containing 0.05% w/v collagenase and 5mM CaCl2 with recirculation for 10 min, and with 100 ml HEPES buffer (pH 7.6) containing 0.1% w/v collagenase and 5 mM CaCl2 with recirculation for about 20 min. The buffers were maintained at 37 oC and continuously gassed with O2 (medical quality). After the collagenase perfusion, the liver tissue was gently teared apart. The loosened mass was passed through 250 uM nylon filter. The filtrate was cooled on ice and then centrifuged at 40 xg for 5 min at 4 oC. The resultant pellet was resuspended in cold HEPES-TES buffer solution (pH 7.6) containing 2% albumine (incubation buffer), and recentrifuged. The pellet was resuspended in cold incubation buffer and incubated in a shaking waterbath (120 rpm) at 37 oC for 15 min. Then the cell suspension was passed through 100 uM nylon filter. Then the viability of the hepatocytes was determined by trypan-blue

exclusion. The hepatocyte suspension was diluted to a density of 10 x 10^6 viable cells/ml.

Preparation of S9mix

The human liver S9mix was prepared as follows. A part of the human liver sample (obtained from the same liver sample, as would be used for the isolation of hepatocytes) was cut into small pieces and immediately placed in liquid nitrogen. After 10 min the pieces were transferred to the freezer (-80 oC) and stored until used. The froozen liver pieces were then minced with a Waring Blendor mixer and subsequently three volumes of ice cold 0.15 M KCl were added and the mixture was homogenized in a Elvehjem-Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 9000 xg for 20 min at 4 oC. The supernatant (S9) was transferred into cryopreservation vials and placed in liquid nitrogen and subsequently stored at -80 oC. All manipulations were performed under sterile conditions. The S9mix used for the mutagenicity assay contained 0.1 ml S9 per ml. The concentration of NADP and G-6-P in the S9mix was 4 mM and 5 mM, respectively.

Mutagenicity assays

Salmonella/hepatocyte suspension assay

The Salmonella/hepatocyte assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile scintillation vials (20 ml), each containing 387.5 ul of hepatocyte suspension (10 x 10^6 viable cells/ml), 0.1 ml of an exponential-growing culture of Salmonella typhimurium (about 2.0 x 10^9 bacteria/ml) and 12.5 ul of a solution of the compound under test, were incubated in triplicate at 37 oC for 2 h. DMN, DEN, and CP were dissolved in saline and sterilized by filtration. The other test compounds were dissolved in dimethylsulfoxide. The mutagenicity

of DMN and DEN appeared effectively inhibited when dissolved in dimethylsulfoxide instead of saline (unpublished data). After incubation, each mixture was plated with a molten top agar containing biotin (no histidine). The number of revertant colonies was counted after 48 h of incubation at 37 oC.

Salmonella/hepatic S9 plate assay

The Salmonella/S9mix plate assay was performed according to the assay described by Ames et al. (1975) for rat liver S9mix, except that exponential growing cultures of Salmonella typhimurium strains hisG46, TA1535, TA1538 and TA100 were used. We used Oxoid nutrient broth instead of Difco nutrient broth . The concentrations of the test substances in both mutagenicity assays, were chozen so that the concentration in the top agar layer in the S9 plate assay approximately equals the concentration in the hepatocyte suspension assay.

Cytochrome-P450 content

The cytochrome-P450 content of the isolated hepatocytes was measured with the cytochrome-P450 CO-difference spectra after reduction with sodium dithionite according to the method of Omura and Sato (1964). Prior to the measurement of the cytochrome-P450 content of the isolated hepatocytes, the hepatocyte suspension was sonicated twice for 20 sec with an interval of 30 sec in an MSE Ultrasonic Disintegrator (100W) at maximal energy. During this step the suspension was cooled on ice.

Measurement of the metabolism of benzo(a)pyrene by rat and human liver S9

The metabolism of B(a)P by rat and human liver S9mix was measured by use of high pressure liquid chromatographic determination of ether-soluble metabolites of B(a)P. The rat liver S9 was prepared from the pooled livers of four non-induced rats, as described by Ames et al. (1975). The S9 was stored at -80 oC. The rat liver S9mix contained NADP (4mM), G-6-P (5mM) and 0.1 ml S9 per ml. B(a)P was incubated with rat or human liver S9mix as follows. In a shaking waterbath (210 rpm) closed vials (inner diameter 30 mm), each containing 1.95 ml S9mix and 50 ul of a solution of B(a)P in dimethylsulfoxide (final concentration of B(a)P in the reaction mixture was 25 uM) were incubated at 37 oC for 2 h. After incubation each mixture was cooled on ice. Then the 2 ml mixtures were extracted with 2x5 ml ether. The organic layers were collected and evaporated to dryness at 30 oC under a constant flow of nitrogen. The residue was dissolved in 2 ml methanol and HPLC-analysed, according to a procedure described by Jongeneelen et al. (1985).

RESULTS

Cytochrome-P450 content and viability of isolated human hepatocytes

Table I shows the cytochrome-P450 content and the viability of the isolated human hepatocytes. Great differences in the cytochrome-P450 content of the isolated hepatocytes were observed between the four subjects, ranging from 85 to 360 pmol/10^6 cells. The viability of the isolated hepatocytes was in these four cases about 80%, as judged with trypan-blue exclusion.

TABLE I

CYTOCHROME-P450 CONTENT AND VIABILITY OF ISOLATED HUMAN HEPATOCYTES

Subject	Age	Sex	Cytochrome-P450 content (pmol / 10^6 cells)	Viability (%)*
I	19	F	125	79
II	20	M	85	81
III	22	M	120	82
IV	51	M	360	80

* Percent of cells excluding Trypan-Blue (0.12 % w/v).

Mutagenicity in the Salmonella/hepatocyte suspension assay and Salmonella/hepatic S9 plate assay

Arylamines

The mutagenicity of BZ, 2-AF and 2-AAF towards Salmonella typhimurium strain TA1538 is shown in Figure 1a. These results indicate that BZ is far more effectively activated to mutagenic products in the hepatocyte assay than in the S9 assay. Obvious interindividual differences in mutagenic activation of BZ are seen in the hepatocyte assay, whereas these differences are almost absent in the S9 assay. 2-AF and 2-AAF are activated less effective with hepatocytes from subject 3 compared with 2 and 4, whereas in the S9 assay the S9 of subject 3 appeared more active than 2 and 4. For 2-AF interindividual differences are less pronounced in the hepatocyte assay, whereas they are obvious in the S9 assay. In both assays these variations are less obvious for 2-AAF. Remarkable is the high mutagenicity of 2-AF and 2-AAF in the S9 assay at relative very low concentrations.

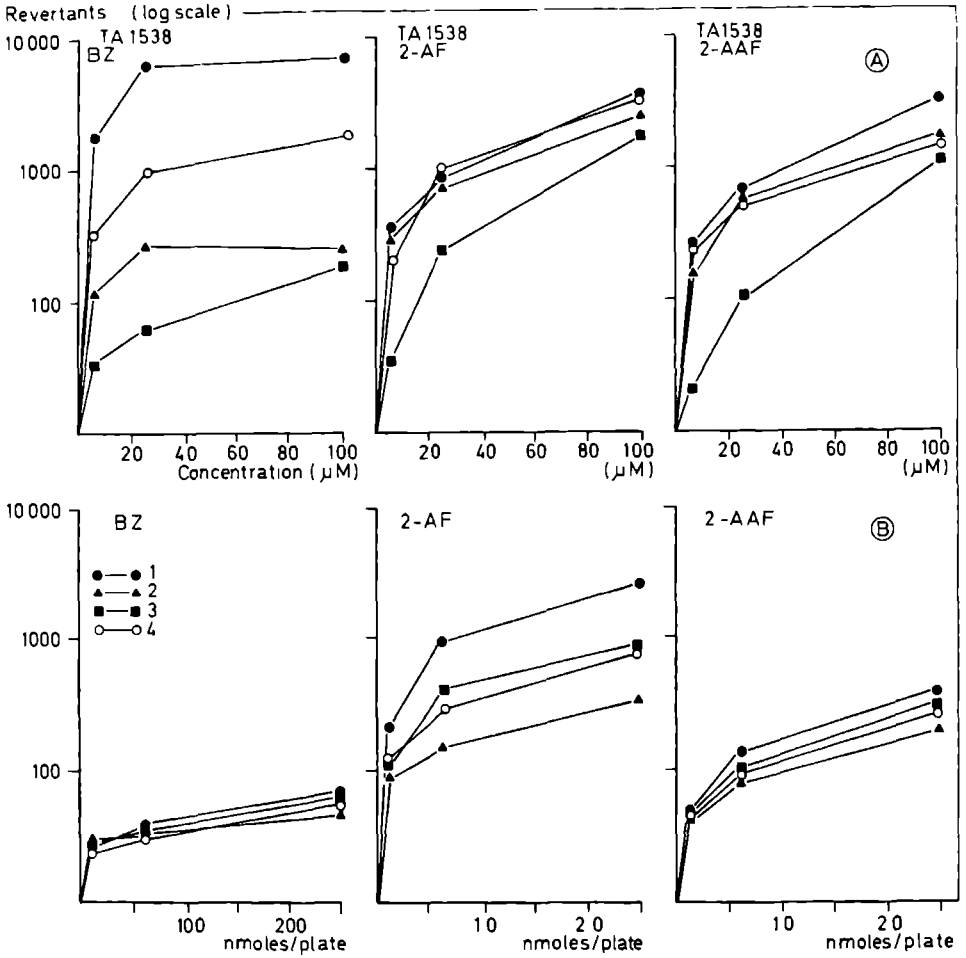

Fig. 1a. Mutagenicity of BZ, 2-AF and 2-AAF towards Salmonella typhimurium strain TA1538, in the Salmonella/hepatocyte suspension assay (A) and in the Salmonella/hepatic S9 plate assay (B). Liver preparations of person 1(●), 2(▲), 3(■) and 4(O). Values represent the mean revertant-number of triplicate determinations. Note the number of revertants is in log-scale.

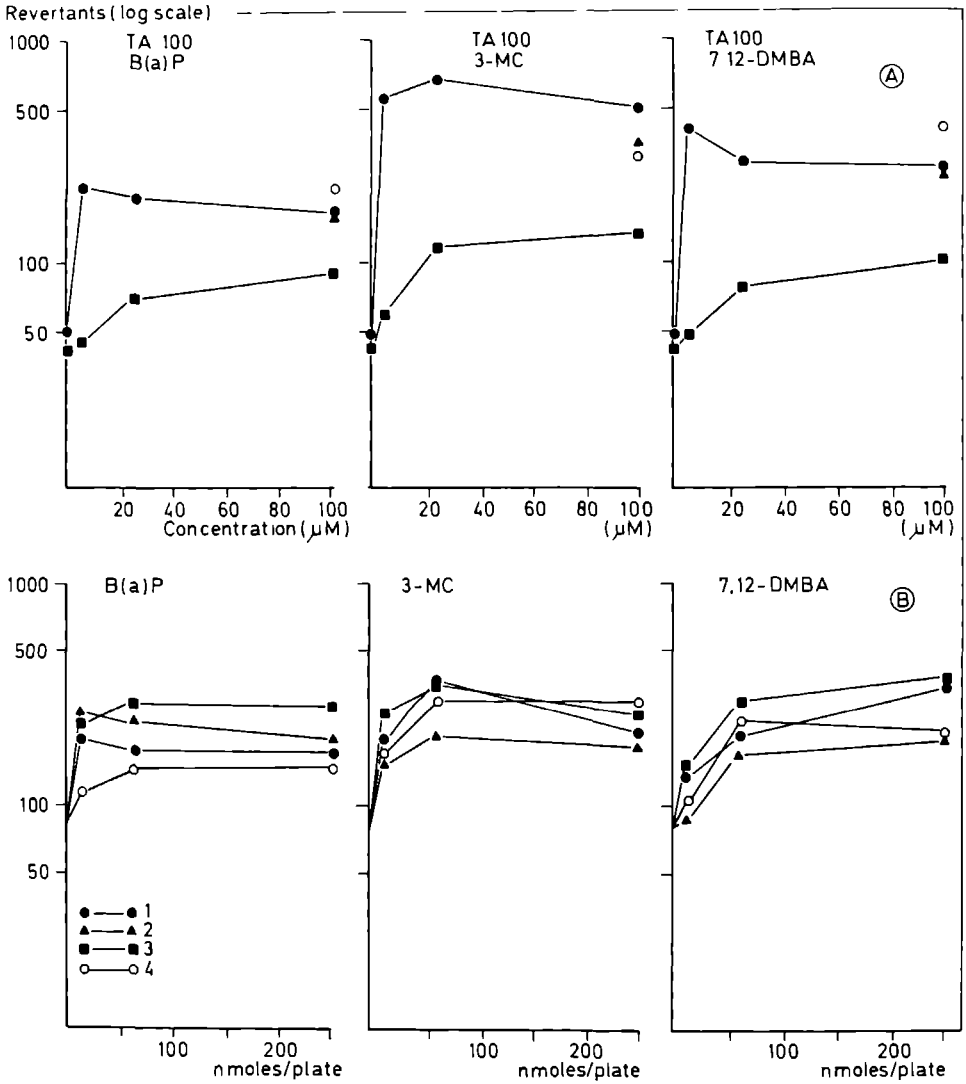

Fig. 1b. Mutagenicity of B(a)P, 3-MC and 7,12-DMBA towards Salmonella typhimurium strain TA100, in the Salmonella/hepatocyte suspension assay (A) and in the Salmonella/hepatic S9 plate assay (B). Liver preparations of person 1(●), 2(▲), 3(■) and 4(○). Values represent the mean revertant-number of triplicate determinations. Note the number of revertants is in log-scale.

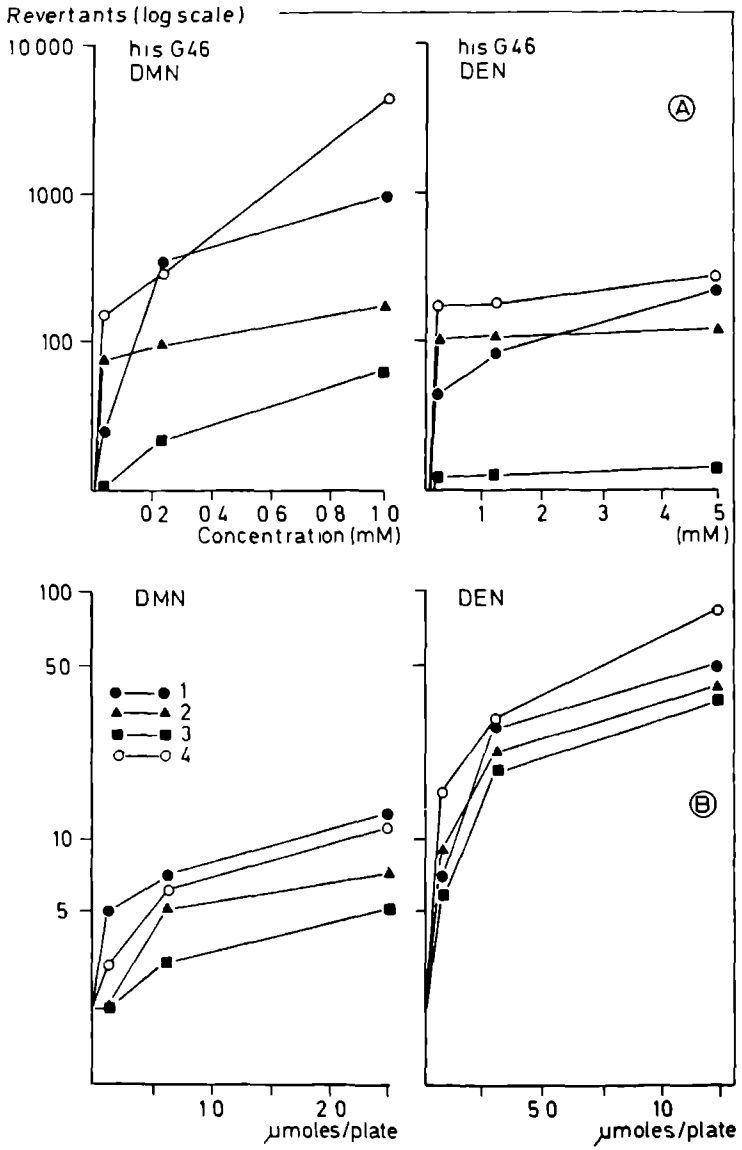

Fig. 1c. Mutagenicity of DMN and DEN towards Salmonella typhimurium strain hisG46, in the Salmonella/hepatocyte suspension assay (A) and in the Salmonella/hepatic S9 plate assay (B). Liver preparations of person 1(●), 2(▲), 3(■) and 4(○). Values represent the mean revertant-number of triplicate determinations. Note the number of revertants is in log-scale.

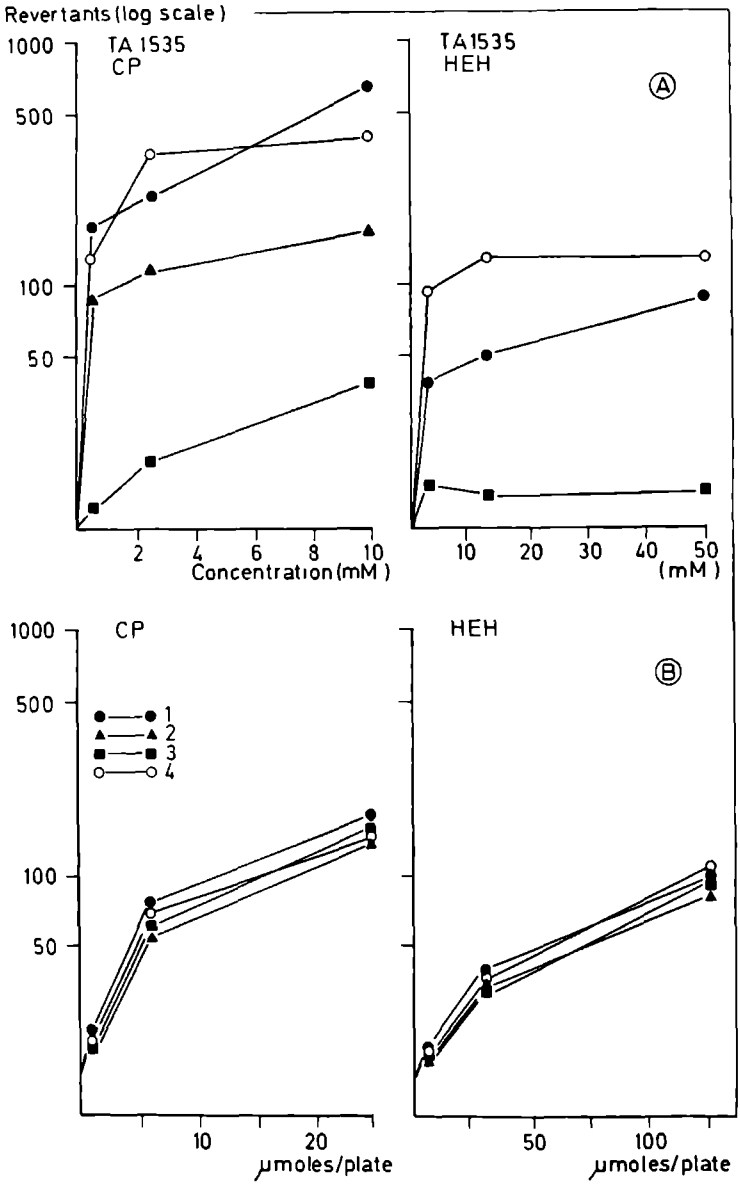

Fig. 1d. Mutagenicity of CP and HEH towards Salmonella typhimurium strain TA1535, in the Salmonella/hepatocyte suspension assay (A) and in the Salmonella/hepatic S9 plate assay (B). Liver preparations of person 1(●), 2(▲), 3(■) and 4(○). Values represent the mean revertant-number of triplicate determinations. Note the number of revertants is in log-scale.

Polycyclic arylhydrocarbons

The mutagenicity of benzo(a)pyrene (B(a)P), 3-methylcholanthrene (3-MC) and 7,12-dimethylbenzanthracene (7,12-DMBA) towards Salmonella typhimurium strain TA100 are presented in Figure 1b. For subject 2 and 4 the three PAH's were only tested at a concentration of 100 uM. All three PAH's showed a relative low mutagenic activity in both assays with liver preparations of all the four individuals. Remarkable is the low mutagenic activity of the three PAH's in the hepatocyte assay for subject 3 compared with 1, 2 and 4, whereas in the S9 assay 3 appeares the more effective in activating the PAH's.

N-nitrosamines

The mutagenicity of DMN and DEN towards Salmonella typhimurium hisG46 is shown in Figure 1c. DMN was highly mutagenic in the hepatocyte suspension assay, whereas its mutagenicity was poorly detected in the S9 plate assay. Also great interindividual differences in the mutagenic activation of DMN were seen in the hepatocyte assay, whereas these differences were absent in the S9 assay. DEN appeared to be highly mutagenic in both assays. Obvious is the low activating potency of subject 3 compared with 1, 2 and 4 in the hepatocyte assay, whereas this difference is less pronounced in the S9 assay.

Cyclophosphamide

The mutagenicity of CP in both assays is presented in Figure 1d. The mutagenicity was determined with Salmonella typhimurium strain TA1535. CP showed mutagenic activity in both assays. However, again interindividual differences were observed in the hepatocyte assay, whereas they were almost not present in the S9 assay.

Hydroxyethylhydrazine

The mutagenicity of HEH is presented in Figure 1d. The mutagenicity of HEH in the hepatocyte assay was only tested with hepatocytes of subject 1, 3 and 4. HEH was mutagenic in both assays. However no interindividual differences were seen in the S9 assay, whereas these variations were clear in the hepatocyte assay.

Comparison of mutagenic activation capacities between the human and rat liver preparations

Table II summarizes some remarkable differences in the mutagenic activity of pregenotoxic agents after activation with human liver preparations on the one hand, and rat liver preparations on the other, using the Salmonella/hepatocyte and the Salmonella/hepatic S9 assays. The results with the rat liver preparations were partly derived from our previous reports (Bos, 1983; Neis, 1985c).

The arylamines BZ, 2-AF and the arylamide 2-AAF appeared to be activated far more effectively to mutagens by human hepatocytes than by rat hepatocytes.

In the S9 plate assay 2-AF and 2-AAF were found to be far more mutagenic when human liver S9 instead of rat liver S9 was used. On the contrary, the polycyclic arylhydrocarbons, in particular B(a)P, were more effectively activated to mutagens, when rat liver S9 was used instead of human liver S9.

For other compounds, like CP, DMN and DEN, no such obvious differences in mutagenic activities were found with rat liver preparations on the one hand, and human liver preparations on the other, in both mutagenicity assays (data not shown).

TABLE II

COMPARISON OF THE CAPACITY TO ACTIVATE PREMUTAGENS BETWEEN HUMAN AND RAT LIVER PREPARATIONS

			hist$^+$ revertants per plate				
			Salmonella/hepatocyte suspension assay				
			Human*				Rat**
		Strain	1	2	3	4	
BZ	(25 uM)	TA1538	6000	270	62	960	103 ± 30
2-AF	(25 uM)	TA1538	810	700	240	990	67 ± 9
2-AAF	(25 uM)	TA1538	660	570	110	490	28 ± 5

			hist$^+$ revertants per plate				
			Salmonella/hepatic S9 plate assay				
			Human*				Rat***
		Strain	1	2	3	4	
2-AF	(2.5 nmol/pl)	TA1538	2400	310	820	705	26
2-AAF	(2.5 nmol/pl)	TA1538	410	195	290	141	12
B(a)P	(65 nmol/pl)	TA100	170	240	290	140	1570
3-MC	(65 nmol/pl)	TA100	350	202	342	295	585
7,12-DMBA	(65 nmol/pl)	TA100	210	176	290	230	595

* Isolated intact hepatocytes, or liver S9-fractions from four different subjects.

** Values represent the mean ± S.E.M. of four separate experiments with hepatocyte preparations of different rats.

*** Values represent the mean revertant number of triplicate plates. The S9-fraction was derived from the pooled livers of four rats.

Comparison of B(a)P hydroxylation by human and rat liver S9 preparations

The question rises whether there is a relationship between the species-dependent difference in mutagenic activation (Table II) and hepatic enzyme activity in vitro.

This was examined for B(a)P. Figure 2 shows the metabolism of B(a)P by human and rat liver S9mix, using HPLC determination of ether-soluble metabolites. In Figure 2 only a representative HPLC-profile (obtained with the S9mix of subject 1) of the four examined subjects is shown. It was observed that non-induced rat liver S9 was far more active in metabolizing B(a)P to ether-soluble metabolites, than the human S9 liver preparations.

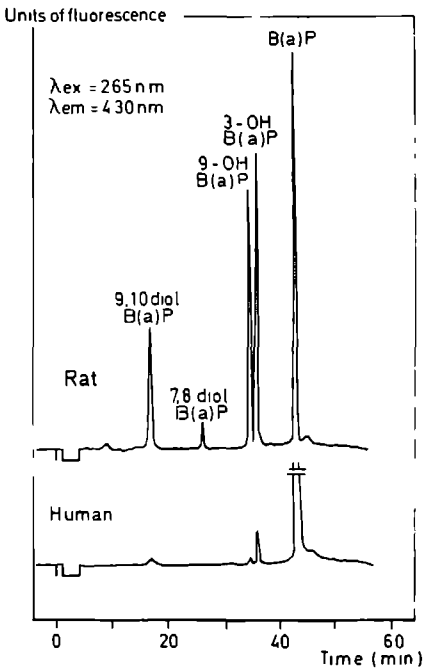

Fig. 2. Comparison of the metabolism of B(a)P by rat and human liver S9mix, using high pressure liquid chromatographic determination of ether-soluble metabolites. Note quantitative differences between rat and man.

For instance, a much higher formation of 3-OH-B(a)P and 9-OH-B(a)P with rat liver S9 was found (Figure 2). In agreement to this a lower amount of unmetabolized B(a)P was found after incubation with rat liver S9 compared with the human liver S9 (Figure 2).

DISCUSSION

In this study, obviously divergent mutagenic values were obtained for the compounds tested, with the human hepatocyte suspension assay on the one hand, and the human S9 plate assay on the other (Figure 1). We recently showed for BZ, 4-AB, 2-AA and DMN, by comparison between mutagenicity values obtained with suspensions of intact rodent hepatocytes and S9 prepared of these cells under comparable experimental conditions, that the observed divergent mutagenicity values with the hepatocyte suspension assay compared to the S9 plate assay, are rather due to intrinsic differences in the activating potential of S9 versus hepatocytes than to methodological differences (Bos, 1983; Neis, 1984). BZ, DMN and DEN appeared to be far more mutagenic in the hepatocyte assay than in the S9 assay (Figure 1a,c). Phillipson et al. (1983) recently also found that BZ was only weakly mutagenic to Salmonella typhimurium TA1538 after metabolic activation with a human S9 liver preparation. These findings are consistent with our previous report that intact rodent hepatocytes are able to activate BZ to mutagenic products, whereas the S9 preparation of these cells was only able to activate BZ when the S9 was supplemented with acetyl-CoA, the cofactor for the N-acetylation reaction (Neis, 1984). We therefore suggest that the lack of N-acetylation in the S9 is responsible for the relative low mutagenicity of BZ in the human liver S9 plate assay, as compared with the human hepatocyte suspension assay.
Glowinski et al. (1978) have reported the existence of the genetic N-acetylation polymorphism of arylamine carcinogens, e.g. BZ and 2-AF, in human liver preparations. The observed

interindividual diffferences in the mutagenic activation of BZ (Figure 1a) may be a reflection of differences in N-acetylation capacity of the isolated hepatocytes of these individuals. We recently showed that N-acetylbenzidine (MABZ) and N,N'-diacetylbenzidine (DABZ) are more mutagenic than BZ itself, when activated by human hepatocytes (Neis, 1985a). Therefore differences in N-acetylation capacity of the hepatocytes, e.g the formation of the more mutagenic MABZ and DABZ, will influence the mutagenicity of BZ. The present results show that the mutagenicity of 2-AF and its acetylated derivative 2-AAF is almost equal after activation with intact hepatocytes (Figure 1a). Therefore differences in N-acetylation capacity between hepatocytes from the four individuals will not influence the mutagenicity of 2-AF or 2-AAF as obvious as BZ.

Interindividual differences were also found for other compounds e.g. DMN, DEN, CP and HEH in the hepatocyte assay (Figure 1c,d). Interindividual variations in the activation of genotoxic compounds by human liver preparations have also been reported by other investigators (Dybing, 1979; Felton, 1984; McManus, 1983; Moore, 1984; Strom, 1982 and 1983). The observed interindividual variations may reflect differences in hepatic drug metabolizing enzymes, that are known to be affected by genetic factors, sex, age, and differences in exposure to inducers like, alcohol, smoking-habbits, diet etc. Obvious is the relative high mutagenic activating capacity of the hepatocytes of subject 1 (Figure 1). This person was known to be an heavy drinker. We have recently shown that hepatocytes isolated from rats pretreated with ethanol, converted certain premutagens more effectively to mutagenic products than hepatocytes from control rats (Neis, 1985c).

Also obvious interindividual differences in the cytochrome-P450 content of the isolated human hepatocytes were observed ranging from 85 to 360 pmol/ 10^6 cells (Table I). Dybing et al. (1979) and Strom et al. (1982) have also reported interindividual variations in the cytochrome-P450 content of human liver preparations. Singer and Grundberger (1983), reported that investigations on cytochrome-P450 from human liver have shown,

that fractions isolated from different individuals have significant different subunit molecular weights. These investigations also showed, that human cytochromes-P450 forms a family of isoenzymes that are distinct from the cytochromes P-450 isolated from experimental animals.

The mutagenicity data of several compounds mediated by human hepatocytes or S9 were compared with those obtained with rat liver preparations. Remarkable differences in the mutagenicity of several compounds were seen after activation with rat liver preparations on the one hand, and human liver preparations on the other (Table II). For instance 2-AAF appeared far more effectively activated to mutagenic products by human hepatocytes or S9, than by rat liver preparations. This may be a reflection of the reported much higher N-hydroxylation of 2-AAF by human than by rat liver cytochromes-P450 (Boobis, 1984). Felton et al. (1984) have reported that human liver microsomes were far more efffective in the cytochrome-P448 dependent activation of certain mutagens in cooked ground beef than microsomes from non-induced rat or mouse liver. Recently, Beaune et al. (1985) reported that the arylamines 2-AA and 2-AF were more mutagenic in the presence of human liver preparations than rat liver preparations in the Ames-assay.

In contrast, for B(a)P a much lower mutagenicity was observed with human liver S9 compared with rat liver S9 (Table II). This may be due to the higher activating capacity of the rat liver S9. Non-induced rat liver S9 appeared to be far more active in metabolizing B(a)P to ether-soluble metabolites, than the human liver S9 (Figure 2). A much greater formation of 3-OH-B(A)P, a metabolite reported as marker for the mono-oxygenase activity, an essential step in the metabolic activation of B(a)P to genotoxic products, was observed with rat liver S9 (Figure 2). Beaune et al. (1985), reported that the polycyclic arylhydrocarbon 3-MC was less mutagenic in the presence of human than rat liver S9 in the Ames-assay. Apart from differences in mutagenic activation between rat and human liver preparations, for some compounds, e.g. CP, DMN and DEN great similarities between the rat and human liver preparations were found (data not shown, see for

comparison Bos et al. 1983 and Neis et al. 1985b).
The observed differences in the ability to activate various mutagens between cellular and subcellular liver preparations on the one hand and between man and rat on the other, indicates that critical toxification or detoxification pathways may easily be overlooked in studies comprising rat S9 liver preparations only. Application of isolated human hepatocytes can be valuable to obtain more insight in this problem.

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Chapter VIII

MUTAGENICITY TOWARDS SALMONELLA TYPHIMURIUM OF SOME KNOWN GENOTOXIC AGENTS, ACTIVATED BY ISOLATED HEPATOCYTES OF MONKEY (MACACA FASCICULARIS) COMPARISON WITH ISOLATED HUMAN HEPATOCYTES

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SUMMARY

This paper describes some striking differences between isolated monkey and human hepatocytes in their capacity to activate some known genotoxic agents to products mutagenic towards Salmonella typhimurium.
Isolated monkey hepatocytes, in contrast to human hepatocytes, appeared to activate benzidine (BZ), N-acetylbenzidine (MABZ), N,N'-diacetylbenzidine (DABZ), 2-aminofluorene (2-AF) and 2-acetylaminofluorene (2-AAF) poorly. With monkey hepatocytes BZ was slightly more mutagenic than DABZ, whereas with human hepatocytes DABZ was more active than BZ. N-nitrosodimethylamine (DMN) and N-nitrosodiethylamine (DEN) were also found to be poorly mutagenic when activated by monkey hepatocytes, unlike the human hepatocytes. However, the polycyclic arylhydrocarbons benzo(a)pyrene (B(a)P) and 7,12-dimethylbenzanthracene (7,12-DMBA) were highly active in the presence of monkey hepatocytes, unlike the human hepatocytes. A metabolic study showed that monkey liver preparations seem to possess a higher mono-oxygenase activity towards B(a)P than human liver preparations.

INTRODUCTION

During the last few years isolated hepatocytes have been increasingly used in combination with Salmonella typhimurium to study the potential genotoxicity of suspected chemicals (Bos, 1983; Langenbach, 1983; Malavielle, 1983; Neis, 1984 and 1985a,b,c; Rumruen, 1984). The Salmonella/hepatocyte assay combines intact cel metabolism (in a cell type with the broadest capability to metabolize foreign compounds) with bacterial mutagenicity as endpoint for genotoxic activity.
We recently reported substantial interspecies differences between isolated rat, dog and human hepatocytes in their capacity to

activate certain chemicals to mutagens (Neis, 1984 and 1985a,b,c). The question arises whether such differences also exist between liver cells from primate species.
This paper presents a comparison of the mutagenicity of some known genotoxic agents measured towards Salmonella thyphimurium after metabolic activation by isolated hepatocytes of monkey (Macaca fascicularis) and man.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: BZ from Merck (Darmstadt, FRG); MABZ and DABZ were from ICN Pharmaceuticals, Inc. (Plainview, NY, USA) and were purified by recrystallization (purity>97%, checked with HPLC); 2-AF, 2-AAF, DMN, B(a)P from Aldrich Europe (Beerse, Belgium); DEN from Aldrich Chemical Co. Inc. (Milwaukee, USA); 7,12-DMBA was from Eastman Kodak Company. Collagenase (from Clostridium histolyticum) was from Boehringer (Mannheim, FRG). All other chemicals used were of high purity.

Preparation of isolated hepatocytes

Monkey hepatocytes

The monkeys used for the isolation of hepatocytes were all male monkeys, and all were about fourtheen years old except monkey I who was about seven years old.
The isolated monkey hepatocytes were prepared under sterile conditions with a two-step collagenase perfusion technique according to the procedure previously described for the isolation of dog hepatocytes (Neis, 1985a), with slight modifications.

Monkeys (Macaca fascicularis), were anesthesized and heparinized. The portal vein was cannulated, the bile duct ligatured, whereafter the liver was perfused in situ with 200 ml of saline so that the liver appeared to blanch totally. Then the liver was removed from the body and transferred to a perfusion apparatus. Approximately 2 liters of a Ca2+-free HEPES buffer (pH 7.4) containing 0.5 mM EGTA, was pumped through the liver at a rate of 100-150 ml/min. Next, 200 ml of HEPES buffer (pH 7.6) containing Ca2+ (5 mM) and 0.05% w/v collagenase with recirculation for 10 min, and 100 ml of the same buffer containing 0.1% w/v collagenase with recirculation for 20 min, was pumped through the liver. After this perfusion, the loosened liver tissue was carefully teared apart and diluted with HEPES-TES buffer containing 2% albumine, and the obtained crude cell suspension was filtered through 250 um nylon mesh and the filtrate centrifuged. The cell pellet was resuspended in HEPES-TES buffer containing 2% w/v albumin. After incubation of this liver cell suspension in a shaking waterbath (120 rpm) at 37 oC for 20 minutes, filtration, gentle centrifugation and cell washing, the final pellet was resuspended in a HEPES-TES buffer solution (pH 7.6) containing 2% albumin. The viability (as judged by trypan-blue exclusion) ranged from 83-93%.

Human hepatocytes

Human hepatocytes were isolated from postmortal human liver tissues, obtained from kidney transplant donors, as previously described (Neis, 1985a,c)

Mutagenicity assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile scintillation vials (20 ml) each containing 387.5 ul of hepatocyte suspension (1 x 10^7 viable cells/ml), 0,1 ml of an

exponential-growing suspension of Salmonella typhimurium (about 2 x 10^9 bacteria/ml) and 12.5 ul of a solution of the compound under test, were incubated in triplicate at 37 oC for 2 h. DMN and DEN were dissolved in saline and sterilized by filtration. The other test compounds were dissolved in dimethylsulfoxide (DMSO). After incubation, each mixture was plated with a molten top agar containing biotin (no histidine). The number of revertant colonies was counted after 48 h of incubation at 37 oC.

Measurement of the metabolism of B(a)P by monkey and human liver S9 preparations

The metabolism of B(a)P by monkey and human liver S9mix (9000 xg liver supernatant supplemented with a NADPH regenerating system) was measured by use of high pressure liquid chromatographic (HPLC) determination of ether-soluble metabolites of B(a)P as previously described (Jongeneelen, 1985; Neis, 1985c). B(a)P (25 uM) was incubated with the S9mix for a period of 2 h and subsequently metabolites were extracted with ether. Identification of metabolites was done by comparison of retention times with reference B(a)P-metabolites. The monkey liver S9mix was prepared in the same manner as the human liver S9mix, as previously described (Neis, 1985c).

RESULTS

Mutagenicity of BZ, MABZ and DABZ

Table I shows the mutagenic activity of BZ, MABZ and DABZ towards Salmonella typhimurium strain TA1538 after metabolic activation with isolated hepatocytes of monkey I, II and III. The hepatocytes of these animals appeared to be capable of activating all three compounds to mutagenic products. With all three hepatocyte preparations, MABZ appeared to be the most mutagenic,

whereas BZ was slightly more active than DABZ. Among these monkeys slight interindividual differences in activating capacity of the isolated hepatocytes were seen.

With human hepatocytes a much higher mutagenicity of these compounds was found. It is striking that with the human hepatocytes, unlike the monkey hepatocytes, DABZ apeared far more mutagenic than the parent compound BZ.

TABLE I

MUTAGENIC EFFECTS ON SALMONELLA TYPHIMURIUM TA1538 OF BZ, MABZ AND DABZ, ACTIVATED BY ISOLATED HEPATOCYTES OF MONKEY (*Macaca fascicularis*) AND MAN

		hist$^+$/revertants per plate			
		Monkey*			Human*
		I	II	III	
BZ	5 (uM)	11	11	7	628 ± 313
	25 (uM)	20	17	11	1738 ± 1091
	100 (uM)	101	42	49	2034 ± 1183
MABZ	5 (uM)	23	9	12	1310
	25 (uM)	78	17	16	4130
	100 (uM)	357	49	72	5225
DABZ	5 (uM)	8	7	7	1200
	25 (uM)	16	10	9	3760
	100 (uM)	40	20	21	4210

Control revertant number was 8 ± 1.

* For monkey, values are the mean revertant number of triplicate incubations.

** For human, values for BZ are the mean ± S.E.M. of experiments with hepatocyte preparations of five different human subjects. Values for MABZ and DABZ are obtained with hepatocytes of one human subject.

TABLE II

MUTAGENIC EFFECTS ON SALMONELLA TYPHIMURIUM OF 2-AF, 2-AAF, B(a)P, 7,12-DMBA, DMN AND DEN, ACTIVATED BY ISOLATED HEPATOCYTES OF MONKEY (*Macaca fascicularis*) AND MAN

Tester Strain	TA1538				TA100				hisG46				
Compound	2-AF		2-AAF		B(a)P		7,12-DMBA			DMN		DEN	
Species	M	H	M	H	M	H	M	H		M	H	M	H
control	8	9± 2	8	9± 2	56	44± 6	56	44± 6	control	2	2± 1	2	2± 1
5 (uM)	14	221± 68	20	168± 52	164	130±100*	99	219±180*	50 (uM)	8	64± 31	9	nt
25 (uM)	20	685±159	27	456±120	530	125± 65*	318	175±105*	250 (uM)	10	189± 78	13	84± 36
100 (uM)	49	2825±416	29	1900±475	2620	160± 29	623	254± 61	1000 (uM)	12	1369±997	17	90± 35

For monkey (M), values are mean revertant number of triplicate incubations with isolated hepatocytes of monkey IV.

For human (H), values are mean revertant number ± S.E.M. of experiments with hepatocyte preparations of four different human subjects, except * are the mean ± S.E.M. of hepatocyte preparations of two human subjects.

nt = not tested.

Mutagenicity of 2-AF, 2-AAF, B(a)P, 7,12-DMBA, DMN and DEN

Table II shows the mutagenicity of genotoxic compounds of different chemical classes towards Salmonella typhimurium after activation by isolated hepatocytes of monkey IV and man. The dose-responses presented in Table II show that all the tested compounds were activated to mutagens, by isolated hepatocytes of monkey IV.

The arylamines 2-AF and 2-AAF and the nitrosamines DMN and DEN were poorly activated by the monkey hepatocytes, in comparison to the values obtained with human hepatocytes. In contrast, the reverse was found for the polycyclic arylhydrocarbons B(a)P and 7,12-DMBA. These compounds appeared highly mutagenic towards Salmonella typhimurium TA100 when activated with the monkey hepatocytes.

Metabolism of B(a)P

In a previous study (Neis, 1985c) we observed a relatively weak mutagenic activity of B(a)P towards Salmonella typhimurium TA100, after metabolic activation with isolated hepatocytes of four human subjects. In that investigation a weak metabolic activation capacity of the human hepatocytes towards B(a)P, as measured by a relatively low formation of ether-soluble B(a)P metabolites, e.g. 3-OH B(a)P, after incubation of human S9mix with B(a)P was suggested as the reason for the observed low mutagenicity of B(a)P.

Therefore, the capability of monkey S9mix to metabolize B(a)P was measured using HPLC determination of ether-soluble metabolites. Figure 1 shows the metabolite profile obtained with the S9mix of monkey IV and a representative human liver S9mix.

In the presence of the monkey liver S9mix the conversion of B(a)P to 9-OH-B(a)P and 3-OH-B(a)P proceeded more effective than with the human liver preparation. In agreement to this a lower amount of unmetabolized B(a)P was found after incubation with the monkey S9mix compared with the human S9mix (Figure 1).

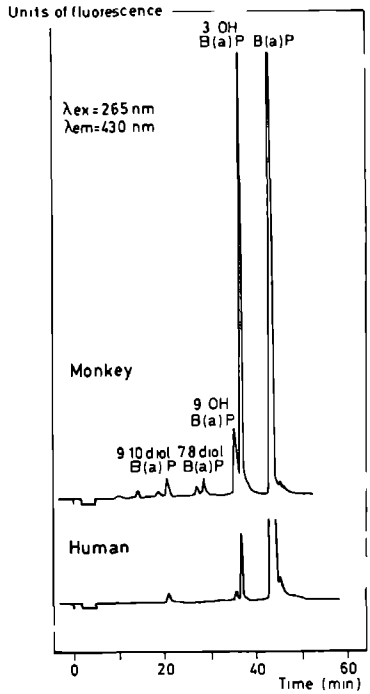

Fig. 2. Comparison of the metabolism of B(a)P by monkey and human liver S9mix, using high pressure liquid chromatographic determination of ether-soluble metabolites. Note quantitative differences between monkey and man.

DISCUSSION

The isolated monkey hepatocytes appeared to be capable of activating all of the tested compounds to products mutagenic towards Salmonella typhimurium. However, great differences in the mutagenic potency between chemical classes were observed. BZ, MABZ and DABZ were found to be poorly activated by monkey hepatocytes. MABZ appeared the most mutagenic (Table I). With human hepatocytes BZ, MABZ and DABZ were highly mutagenic towards Salmonella typhimurium TA1538, showing MABZ and DABZ equally active but far more mutagenic than the parent compound BZ (Table I). 2-AF and 2-AAF were also only weakly mutagenic

towards TA1538 in the presence of monkey hepatocytes (Table II). In contrast, with isolated hepatocytes of four human subjects, 2-AF and 2-AAF appeared to be relatively highly mutagenic. The reason for these interspecies differences may be attributed to a lower rate of N-hydroxylation (an essential step in the activation of arylamines and arylamides) in the monkey hepatocytes compared with human hepatocytes.

The nitrosamines DMN and DEN were also found only weakly mutagenic in the presence of monkey hepatocytes, whereas these compounds were highly mutagenic when activated by isolated human hepatocytes. In contrast, the polycyclic arylhydrocarbons B(a)P and 7,12-DMBA were found highly mutagenic after metabolic activation with monkey hepatocytes, unlike human hepatocytes. Presumably, the higher mutagenicity of B(a)P with monkey than with human hepatocytes is due to a higher activating capacity of the monkey hepatocytes. The monkey S9 liver preparation appeared to be more active in metabolizing B(a)P to ether-soluble metabolites, than human liver S9mix (Figure 1). Much higher amounts of 3-OH-B(a)P, a metabolite reported as marker for mono-oxygenase activity an essential step in the metabolic activation of B(a)P to genotoxic products, were found after incubation of B(a)P with monkey liver S9mix (Figure 1). Monkey liver has been reported to possess a relatively low GSH-transferase activity (Chasseaud, 1977). Therefore, a low detoxification rate may also contribute to the high mutagenicity of B(a)P in the presence of monkey hepatocytes. After incubation of B(a)P with the monkey S9 liver preparation 7,8-diol-B(a)P, 9,10-diol-B(a)P, 9-OH-B(a)P and 3-OH-B(a)P were detected (Figure 1). Hundley and Freudenthal (1977) have reported these compounds as metabolites of B(a)P after incubation with liver microsomes of rhesus monkey (Macaca mulatta); 3-OH-B(a)P was reported to be the major metabolite. These authors reported only slight interindividual differences between the monkeys in the formation of these metabolites.

The obvious divergent mutagenicity data obtained with isolated monkey hepatocytes as compared with human hepatocytes are somewhat surprising, since one should expect that a subhuman

primate species like the Macaca fascicularis would resemble the human species more closely than other laboratory animals.

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Chapter IX

MUTAGENICITY AND DNA-EXCISION REPAIR INDUCED BY ISONIAZID AFTER METABOLIC ACTIVATION BY ISOLATED HUMAN AND RAT HEPATOCYTES

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SUMMARY

The mutagenic potency of isoniazid (a widely used antitubercular drug) towards Salmonella typhimurium strain hisG46 was studied in the Salmonella/hepatocyte suspension assay, comprising isolated human or rat hepatocytes as metabolic system. The potency of isoniazid to induce DNA-excision repair in these hepatocytes was also measured.
With rat hepatocytes isoniazid appeared to be only weakly mutagenic and did not induce significant increases in hepatocellular DNA-excision repair. With isolated hepatocytes of two human subjects isoniazid appeared also only weakly mutagenic. However, with hepatocytes of two other human subjects isoniazid was found higly mutagenic. Comparable results were obtained for the induction of hepatocellular DNA-excision repair.

INTRODUCTION

Isoniazid (isonicotine acid hydrazide; INH) has been widespread used for many years for the therapeutic and prophylactic treatment of tuberculosis. Many studies have indicated that prolonged therapy with this drug may lead to severe liver injury in humans. In mice INH has been reported to induce lung tumors (IARC Monograph, 1974). Its carcinogenic activity in rats is however inconclusive or negative (Jansen, 1980).
In many species the major route of metabolism of INH involves acetylation to acetylINH. Large differences between individuals in the acetylation of INH are reported (Ellard, 1975; Evans, 1964; Mitchell 1975a; Nelson, 1976; Weber, 1983). This variation is due to the genetic difference in cytosolic N-acetyltransferase activity, primarily located in the liver. Metabolic studies have shown, that people having a rapid acetylation phenotype are exposed to much more acetylINH and acetylhydrazine than people having a slow acetylation phenotype (Ellard, 1975; Mitchell,

1975a). An increasing number of evidence exist, that INH related liver injury is caused by the metabolite acetylhydrazine, formed by metabolic hydrolysis of acetylINH. (Gatehouse, 1984; Mitchell, 1975a; Nelson, 1976; Timbrell, 1980; Tomasi, 1983). The acetylhydrazine formed, has been reported to be metabolized by the cytochrome-P450 enzym system to reactive acylating species capable of reacting covalently with tissue macromolecules (Nelson, 1976; Timbrell, 1980; Tomasi, 1983).
We recently suggested that isolated human hepatocytes may be a very good model for the study of the potential genotoxicity of compounds to humans (Neis, 1985a and 1985c). In the present investigation the ability of INH to evoke hepatocellular DNA-excision repair and bacterial mutagenicity was studied using isolated hepatocytes from rats and from four human subjects. The N-acetylating activity of these cells was studied in relation to their capacity to bioactivate INH.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Isoniazid (isonicotinic acid hydrazine; INH) was from OPG (Utrecht, The Netherlands); collagenase (from Clostridium histolyticum) was from Boehringer (Mannheim, FRG); Eagle's minimal essential medium including Hank's salts, and foetal calf serum were from Biomed; (3H)-Thymidine (3H-TdR), spec. act. 26 Ci/mmol was from The Radiochemical Centre (Amersham, UK).
All other chemicals used were of high purity.

Preparation of isolated rat and human hepatocytes

Isolated rat hepatocytes were prepared by a two-step collagenase perfusion technique as previously described (Neis, 1984 and

1985b). The human hepatocytes were prepared from liver samples (200-400 g) obtained from kidney transplant-donors killed in a traffic accident. The human hepatocytes were isolated by a modified two-step collagenase perfusion-technique as previously described (Neis, 1985a and 1985c). All manipulations were carried out under sterile conditions.
The rat and human 9000 xg liver supernatant fractions (S9mix) were prepared as described previously (Neis, 1985c).

Mutagenicity assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 rpm), closed sterile scintillation vials, (20 ml) each containing 387.5 ul of hepatocyte suspension (10 x 10^6 viable cells/ml), 0,1 ml of an exponential-growing suspension of Salmonella typhimurium strain hisG46 (about 2.5 x 10^9 bact./ml) and 12.5 ul of a sterile solution of INH in saline, were incubated in triplicate at 37 oC for 2 h. After incubation, each mixture was plated with a molten top agar containing biotin (no histidine). The number of revertant colonies was counted after 48 h of incubation at 37 oC. The Salmonella/S9mix suspension assay was performed as described for the hepatocyte assay except that liver S9mix was used instead of isolated hepatocytes. The Salmonella/S9mix plate assay (Ames-assay) was performed as previously described (Neis, 1985c).

DNA-repair assay

Hepatocellular DNA-excision repair was measured as 3H-TdR incorporation into DNA by a rapid and simple technique via liquid scintillation counting as described by Brouns et al (1979), with slight modifications. The isolated hepatocytes were resuspended in Eagle's minimal essential medium, supplemented with Hank's salts, 10% foetal calf serum, streptomycin 0.01% and penicillin-G 100 U/ml. In a shaking waterbath (120 rpm), closed scintillation

vials (20 ml) each containing 487.5 ul of hepatocyte suspension (1 x 10^6 viable cells/ml) and 12.5 ul of a solution of INH in saline, were incubated in triplicate at 37 oC for 1 h. To three vials, serving as control, 12.5 ul saline was added. Subsequently, 3H-TdR was added to each vial to a final concentration of 1 uCi/ml, whereafter incubation was continued for 2 h. Next, 2 ml of saline was added to each vial. Each mixture was transferred to centrifuge tubes and centrifuged at 3000 xg for 10 min and the supernatants were carefully decanted. To the cell pellets, 1 ml 6M NaSCN solution was added under vigorous shaking, and were left to stand overnight at room temperature to achieve complete lysis of the cells. Subsequently, after addition of 1 ml carrier DNA solution (0.1 mg DNA/ml), macromolecules were precipitated by addition of 2 ml 20% ice-cold TCA solution under vigorous shaking. After standing at -20 oC for 30 min the tubes were centrifuged at 30 xg for 15 min. The pellets were washed once with 70% ethanol and once with cold 0.5N PCA, after which the precipitated DNA was selectively solubilized with 0.5 ml 0.5N PCA at 80 oC for 60 min. The tubes were then centrifuged at 3000 xg for 15 min, and the supernatants transferred to scintillation vials. After addition of 5 ml Aqua Luma to each vial, the 3H-radioactivity was measured by a Philips liquid-scintillation analyzer (efficiency 39-42%). 2-Aminofluorene a well known precarcinogen was used as a positive control, to ensure that the isolated hepatocytes possessed an adequate metabolic activation and/or DNA-repair activity.

Cytochrome-P450 content

The cytochrome-P450 content of hepatocytes was measured with the cytochrome-P450 CO-difference spectra after reduction with sodiumdithionite, according to the method of Omura and Sato (1964). Prior to the measurement of the cytochrome-P450 content of the isolated hepatocytes, the hepatocyte suspension was sonicated twice for 20 sec with an interval of 30 sec in an MSE

Ultrasonic Disintegrator (100 W) at maximal energy. During this step the suspension was cooled on ice.

N-acetylation assay

The N-acetylation activity of the isolated hepatocytes was measured with benzidine as a model substrate (Glowinsky et al. (1978) have shown that benzidine, a carcinogenic arylamine, was N-acetylated polymorphically by human liver in vitro in a similar fashion as INH).
The following procedure was used. In a shaking waterbath (210 rpm), closed vials, containing 487.5 ul hepatocyte suspension (10 x 10^6 viable cells/ml) and 12.5 ul of a solution of benzidine in DMSO (final concentration 100 uM) were incubated in triplicate at 37 oC during different time intervals. Next, the mixtures were pipetted into cold TCA and after centrifugation the amount of diazotable amine was determined according to the method of Bratton and Marschall (1939). The extent of acetylation was taken as the difference between initial and remaining free amine and expressed as pmol of substrate acetylated per min per 10 cells. No non-enzymatic disappearance of the substrate was observed.

RESULTS

Mutagenicity

The mutagenicity of INH towards Salmonella typhimurium strain hisG46 after activation with isolated rat and human hepatocytes is presented in Figure 1. INH appeared to be only weakly mutagenic when activated with rat hepatocytes.
With human hepatocytes great interindividual differences were observed in the activating potential towards INH.

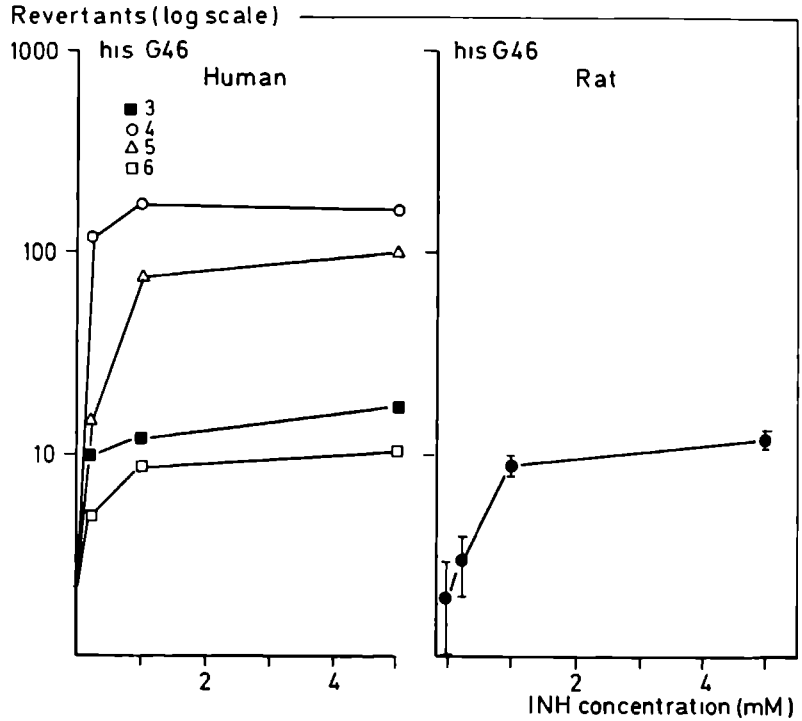

Fig. 1. Mutagenicity of INH towards Salmonella typhimurium strain hisG46 after metabolic activation with isolated human or rat hepatocytes. For human hepatocytes values are the means of determinations in triplicate. For rat hepatocytes values are the means (±SEM) of six separate experiments with hepatocyte preparations of different rats.

The hepatocytes of subject 3 and 6 activated INH only weakly to mutagenic products. The mutagenic values were comparable to those observed with rat hepatocytes. However, a high mutagenic activity of INH was observed with the hepatocytes of subject 5 and, in particular, of subject 4 (Figure 1). Even at relative low concentrations (200 uM) a high mutagenicity (50 times background revertant number) was observed after activation with hepatocytes of subject 4.

The direct mutagenicity of INH and the mutagenicty of INH after metabolic activation with 9000 xg liver supernatant (S9mix) of rats or the four human subjects, was also measured. INH was incubated with Salmonella typhimurium hisG46 in the suspension

assay (up to a concentration of 5mM), or in the plate assay (up to 12.5 umoles/plate), with and without rat or human liver S9mix. In neither case mutagenic activity of INH was found.

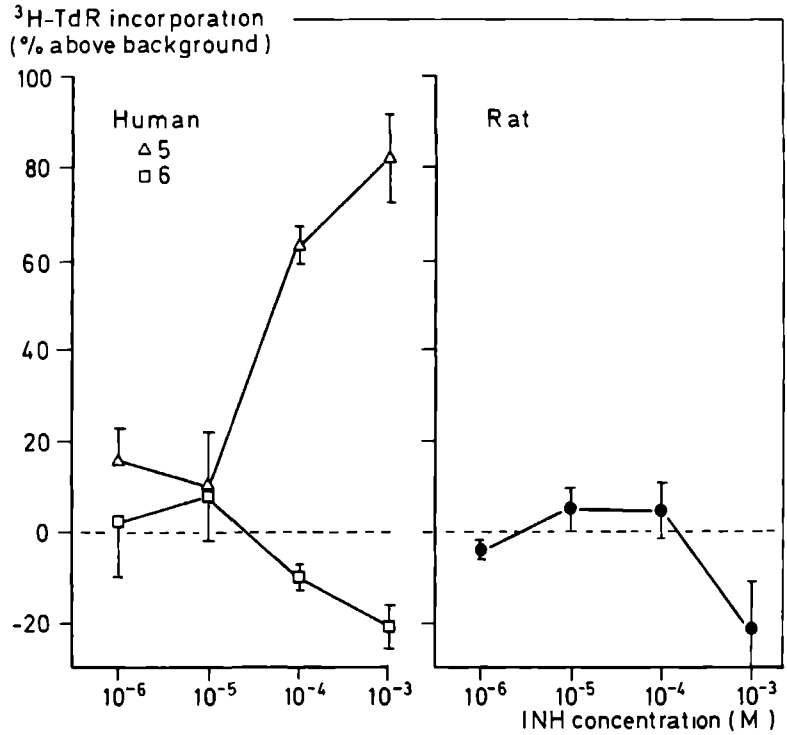

Fig. 2. Incorporation of 3H-TdR into hepatocellular DNA as a result of exposure of isolated human or rat hepatocytes to different concentrations of INH. For human hepatocytes values are the means (±SEM) of triplicate determinations. For rat hepatocytes values are the means (±SEM) of three separate experiments with hepatocyte preparations of different rats.

DNA-repair

DNA-repair was determined with rat hepatocytes and with the hepatocytes of subject 5 and 6. The induction of unscheduled DNA-repair (measured as the incorporation of 3H-TdR into hepatocellular DNA), as a result of exposure of the isolated rat and human hepatocytes to different concentrations of INH are shown in Figure 2.

At the concentrations of INH tested no significant increase in DNA-repair could be detected with rat hepatocytes. The hepatocytes of the two different subjects responded differently to the exposure to INH. No increase in DNA-repair was seen in the hepatocytes of subject 6. Whereas, in the hepatocytes of subject 5 a relatively high increase in DNA-repair was observed.

Cytochrome-P450 content

The cytochrome-P450 content of the isolated hepatocytes of six rats and the four human subjects is shown in Table I. Great differences in the cytochrome-P450 content between the four subjects were seen. The hepatocytes of subject 4 possessed a high cytochrome-P450 content relative to the other three subjects. As was judged from the clinical data this could not be due to induction by drugs given while on live support system. It may be due to induction by prior drug treatment, but this could not be checked.

TABLE I

CYTOCHROME-P450 CONTENT AND N-ACETYLATION IN ISOLATED HUMAN AND RAT HEPATOCYTES

Subject	Age	Sex	Viability* (%)	Cytochrome-P450 content (pmol / 10^6 cells)	N-acetylation** (pmol/min per 10^6cells)
3	22	M	82	120	n.d.
4	51	M	80	360	n.d.
5	46	M	89	154	68
6	16	F	78	112	14
RAT	-	M	95	270 ± 30***	77 ± 8***

* Percent of isolated cells excluding trypan-blue (0.12 % w/v).

** Initial N-acetylation rate of 100 uM benzidine (modelsubstrate).

*** Mean (± S.E.M.) of separate hepatocyte-preparations of six rats.

N-acetylation

The N-acetylation capacity of the isolated hepatocytes from two persons and six rats was determined with benzidine as a substrate (Table I). A great difference was found in the N-acetylating capacity of the hepatocytes of subject 5 compared with subject 6. The hepatocytes of subject 5 possessed an N-acetylation rate five times faster than the hepatocytes from subject 6. The rat hepatocytes appeared to have an N-acetylation rate approximately equal to that of subject 5.

DISCUSSION

Testing of INH in combination with rat hepatocytes revealed that this compound is weakly mutagenic and does not induce a significant increase in DNA-excision repair (Figures 1 and 2). With hepatocytes from human origin (subject 4 and 5), high genotoxic activity was found after exposure to INH, whereas with the cells from subjects 1 and 4 values were obtained that are comparable with those of the rats (Figures 1 and 2). Such variations are well compatible with previous reported differences in hepato-toxicity of INH between rat and man. Thomas et al. (1977) showed by measurement of serum transaminase levels in rats dosed with INH, that the rat is not susceptible to an acute hepatotoxic reaction to INH. Mitchell et al. (1975b) also reported that hepatic necrosis could not be produced in the rat even after lethal INH doses. Probst et al. (1983) reported INH to be also negative in a genetic toxicology animal based test-battery, comprising bacterial-mutation, DNA-repair, L5178Y-mutation and in vivo SCE.

Thomas et al. (1977) found quantitative differences in the metabolism of INH between rats and humans. Their results indicated that the rat is unable to hydrolyse acetylINH to acetylhydrazine as rapidly as humans, thereby explaining the lower toxicity in rat compared to man. All these findings

indicate that the rat may not be a proper model to study the (geno)toxicity of INH to humans.

Several investigations have indicated the importance of the N-acetylation reaction in the bioactivation of INH (Gatehouse, 1984; Nelson, 1976; Timbrell, 1980; Tomasi, 1983). We found that the N-acetylation activity of isolated hepatocytes from two human subjects were widely divergent (Table I). The N-acetylation activity of the hepatocytes of subject 5 was almost five times higher than that of subject 6. A good correlation has been reported between the capacity to acetylate INH in vivo and the in vitro activity of the liver acetylating enzymes (Evans, 1964). In man in vivo as well as in vitro with liver-biopsies, a three-five times faster acetylation of INH, acetylhydrazine and sulfadimide has been reported for rapid acetylators than for slow acetylators (Ellard, 1975). Rat hepatocyte preparations appeared to possess an acetylation activity almost equal to that of the hepatocytes of subject 5. This is well compatible with the results of Thomas et al. (1977) and Ellard et al. (1975) that indicated that INH is acetylated to acetylINH at the same rate in rats as in rapid acetylating humans.

The relation between acetylation polymorphism in man and the toxicity of INH to the liver has been studied at epidemiological, clinical and basic levels. However, considerable controversy has generated from these studies (Weber, 1983). Examination of INH metabolites in humans by Mitchell et al. (1975a) revealed that rapid acetylators are exposed to 46% more acetylhydrazine (proximate genotoxic metabolite) than slow acetylators. Our observation, that with the rapidly acetylating hepatocytes of subject 5 a much higher genotoxicity was found, makes it attractive to assume that rapid-acetylator humans may be at increased risk for INH liver injury. However, because the activation of INH, apart from N-acetylation, also involves cytochrome-P450 mediated metabolism, the observed difference in INH genotoxicity may also be partly due to interindividual variations in the cytochrome-P450 system.

Although liver necrosis presumably due to a reactive metabolite is found in therapy with INH (Lenders, 1983), it is not known

whether or not it is also a carcinogen to humans. However, the present observations on mutagenicity and induced DNA-repair by INH with human hepatocytes might be a reason for concern about a possible risk of neoplasm after INH therapy.

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Chapter X

INFLUENCE OF ETHANOL INDUCTION ON THE METABOLIC ACTIVATION OF GENOTOXIC AGENTS BY ISOLATED RAT HEPATOCYTES

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SUMMARY

The effects of ethanol feeding to rats, over a 6 week period, on the activation of genotoxic compounds of different chemical classes, requiring metabolic conversion to exert their mutagenic activity, were studied in isolated rat hepatocytes. The influence of such treatment on cytochrome-P450 content and N-acetylation in isolated hepatocytes was also investigated.
Benzidine (BZ), dimethylnitrosamine (DMN), diethylnitrosamine (DEN), isoniazid (INH) and cyclophosphamide (CP) were more effectively activated to products mutagenic towards Salmonella typhimurium by hepatocytes from ethanol pretreated rats than by hepatocytes from controls. The mutagenic potency of 2-aminofluorene (2-AF) and 2-acetylaminofluorene (2-AAF) was not influenced by ethanol pretreatment. Ethanol consumption was found to be associated with increased cytochrome-P450 content and enhanced N-acetylation in the isolated hepatocytes.
Our results support the hypothesis that an alteration of the hepatic drug-metabolizing system may be responsible for the ethanol induced increase in susceptibility to certain genotoxic compounds.

INTRODUCTION

Ethanol can induce a variety of metabolic and morphologic changes in both animal and human liver (De Carli, 1967; Elves, 1984; Ishii, 1973; Khanna, 1976; Rubin, 1968; Schnellmann, 1984). Chronic ethanol intake, for instance, alters the hepatic drug-metabolizing system so that certain drugs and endogenous compounds are metabolized more rapidly. Ethanol pretreatment may change the balance between toxication and detoxication, a crucial factor in determining the genotoxic potency of a compound. As a consequence, the potency of agents that become genotoxic as a result of metabolic activation may be altered. Recent findings

indicate that ethanol consumption in rodents results in an increased microsomal capacity to activate a number of pregenotoxic agents (Garro, 1981; Capel, 1979; Smith, 1984).
Isolated hepatocytes have been shown to be a valuable tool for the study of the biotransformation of pregenotoxic agents, particularly those compounds that undergo complex reaction sequences. Metabolism in isolated hepatocytes more closely resembles the biotransformation in the liver in vivo than does metabolism in subcellular liver fractions (Bos, 1982; Glatt, 1981; Billings, 1977). The natural balance between activation and deactivation reactions is better preserved in intact hepatocytes than subcellular fractions, e.g. microsomes.
In the present investigation, isolated rat hepatocytes were used to study the influence of ethanol feeding to rats on the mutagenic activation of genotoxic compounds of different chemical classes, all requiring metabolic conversion to exert their genotoxicity. The influence of ethanol pretreatment on N-acetylation was particularly investigated, because of its importance in the mutagenic activation of some of the compounds studied.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Ethanol used for the treatment of rats was from Nedalco (Bergen op Zoom, The Netherlands); BZ from Merck (Darmstadt, FRG); 2-AF and 2-AAF and DMN from Aldrich Europe (Beerse, Belgium); DEN from Aldrich Chemical Co. Inc. (Milwaukee, USA); CP from Asta (Brackwede, FRG); INH from OPG (Utrecht, The Netherlands). D-biotin, L-histidine-HCl, collagenase type I were obtained from Sigma (St. Louis, Missouri, USA). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain). All other chemicals used were of high purity.

Ethanol pretreatment

Male Wistar rats were fed a standard laboratory diet. The four ethanol treated rats received a 15% (v/v) ethanol solution in place of drinking water for 6 weeks, whereas the four control rats received drinking water. Ethanol treated rats were switched to drinking water 16 h prior to isolation of hepatocytes. Prior to the isolation of hepatocytes, all rats were deprived of food overnight. At the time of use no difference in body weight between the control and the ethanol fed rats was observed.

Preparation of isolated hepatocytes

Hepatocytes were isolated according to a previously described procedure (Neis, 1984). All manipulations were performed under sterile conditions. The final cell suspension was diluted to a density of 10×10^6 viable cells/ml. Trypan-blue exclusion showed the presence of about 95% viable cells for control as well as ethanol pretreated rats.

Mutagenicity assay

The Salmonella/hepatocyte mutagenicity assay was carried out as follows. In a shaking waterbath (210 rpm) closed sterile scintillation vials (20 ml), each containing 387.5 ul of hepatocyte suspension (10×10^6 viable cells/ml), 0.1 ml of an exponential-growing suspension of Salmonella typhimurium (about 2.0×10^9 bacteria/ml) and 12.5 ul of a solution of the compound under test, were incubated in triplicate at 37 oC for 2 h. BZ, 2-AF and 2-AAF were dissolved in dimethylsulfoxide, whereas DMN, DEN, INH and CP were dissolved in saline and sterilized by filtration. After incubation, each mixture was plated with a molten top agar containing biotin (no histidine). The number of revertant colonies was counted after 48 h of incubation at 37 oC.

N-acetylation assay

The rate of N-acetylation in the hepatocyte suspension was measured with p-aminobenzoic acid (PABA) or benzidine (BZ). The procedure was as follows. In a shaking waterbath (210 rpm) closed sterile scintillation vials (20 ml), each containing 487.5 ul of hepatocyte suspension (8 x 10^6 viable cells/ml) and 12.5 ul of a solution of PABA or BZ (4 mM) in dimethylsulfoxide were incubated in triplicate at 37 oC for different time intervals. Next, the concentration of PABA or BZ in these mixtures was measured according to the method of Bratton and Marshall (1939). The disappearance of the substrate remained linear with time up to 45 min incubation.

Cytochrome-P450 content

The cytochrome-P450 content of hepatocytes was measured with the cytochrome-P450 CO-difference spectra after reduction with sodium dithionite, according to the method of Omura and Sato (1964). Prior to the measurement of the cytochrome-P450 content of the isolated hepatocytes, the hepatocyte suspension was sonicated twice for 20 sec with an interval of 30 sec in an MSE Ultrasonic Disintegrator (100W) at maximal energy. During this step the suspension was cooled on ice.

RESULTS

The influence of ethanol on cytochrome-P450 content and N-acetylation in isolated hepatocytes

Table I shows the N-acetylation rate in isolated hepatocytes of four control and four ethanol pretreated rats. The N-acetylation rate of PABA and BZ appeared significantly (Wilcoxon test, $P<0.05$) increased after ethanol pretreatment, by about 45%. The

cytochrome-P450 content of isolated hepatocytes of four control and four ethanol pretreated rats was also compared. The ethanol pretreatment appeared to result in a significant (Wilcoxon test, $P<0.05$) increase in the cytochrome-P450 content of the isolated hepatocytes. The cytochrome-P450 content was increased from 0.26 ± 0.02 (mean SEM) nmoles/10^6 cells to 0.36 ± 0.02 nmoles/10^6 cells.

TABLE I

N-ACETYLATION IN ISOLATED HEPATOCYTES OF CONTROL AND ETHANOL-PRETREATED RATS

Exp.	Substrate	Control	Ethanol	Increase
(1)	BZ	92	142	54 %
(3)	BZ	75	94	25 %
(4)	BZ	65	100	54 %
			Mean	44 %
(2)	PABA	50	83	66 %
(3)	PABA	56	79	41 %
(4)	PABA	46	58	26 %
			Mean	44 %

Values represent the N-acetylation rate in pmol/min per 10^6 cells. Experiment number refers to four separate experiments with hepatocyte preparations from different rats. For both substrates differences in N-acetylation are significant ($P<0.05$, Wilcoxon test).

Mutagenicity

The mutagenicity of 2-AF, 2-AAF, BZ, DMN, DEN, INH and CP towards Salmonella typhimurium after metabolic activation with isolated hepatocytes of control and ethanol pretreated rats is shown in Figure 1.

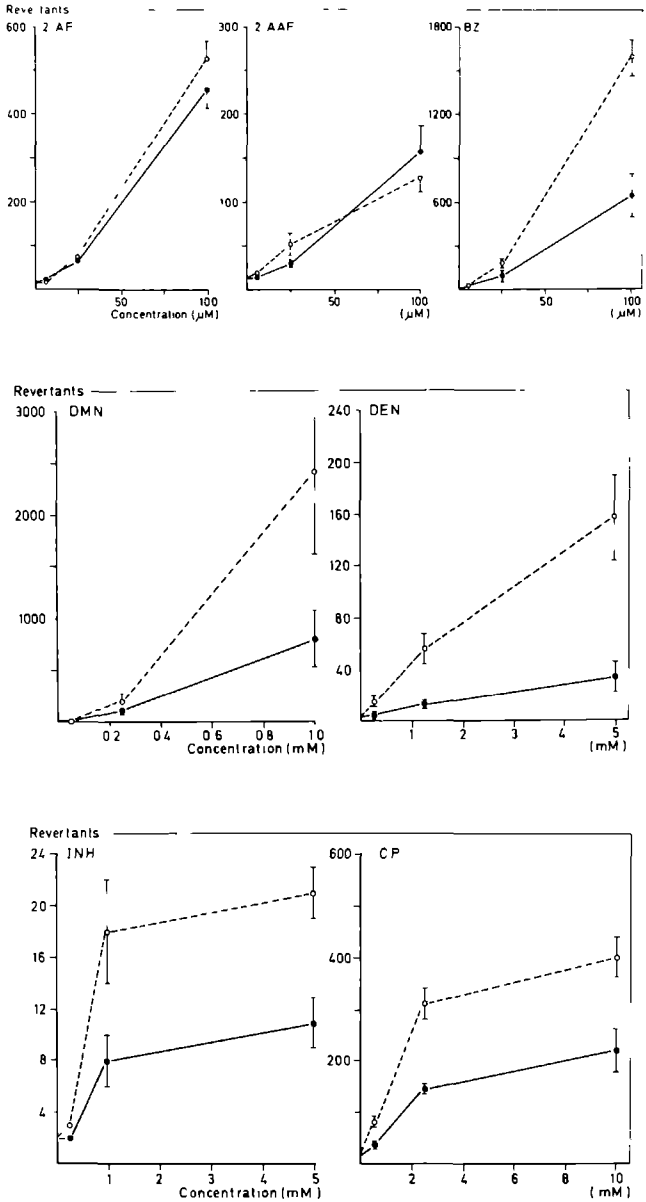

Fig. 1. Mutagenicity of 2-AF, 2-AAF and BZ towards Salmonella typhimurium strain TA1538; of DMN, DEN and INH towards strain hisG46; and of CP towards strain TA1535, after metabolic activation with isolated hepatocytes of control (●—●) and ethanol pretreated rats (O--O). Values represent the mean (±SEM) of four separate experiments with hepatocyte preparations from different rats, each assayed in triplicate.

Ethanol pretreatment resulted in an increase in the mutagenicity of the arylamine BZ, whereas the mutagenicity of 2-AF and 2-AAF was not influenced (Figure 1a).
An increase in mutagenicity was also observed (Figure 1b) for the dialkylnitrosamines DMN and DEN.
Cytostatic CP was also more effectively transformed to products mutagenic to Salmonella typhimurium by hepatocytes of ethanol pretreated rats than by hepatocytes from control rats (Figure 1c). Pretreatment also increased the mutagenicity of INH (Figure 1c).

DISCUSSION

The ability of ethanol to potentiate the toxicity of several drugs is well documented (Embly, 1977; Maling, 1975; Moldeus, 1980; Strubelt, 1978 and 1980). As the most attractive hypothesis to explain this potentiation, these authors suggested that ethanol, by altering the drug-metabolizing system, causes more efficient conversion of the pretoxic agents into highly reactive intermediates.
In our study, ethanol pretreatment resulted in an increase in the cytochrome-P450 content of the isolated hepatocytes. An increase in the hepatic cytochrome-P450 content and enhanced cytochrome-P450 dependent metabolism of several drugs after ethanol treatment is a generally recognized phenomenon. Ethanol pretreatment may increase the content of certain cytochrome-P450 isoenzymes, thereby changing the balance between cytochrome-P450-dependent toxication and detoxication reactions. This may result in potentiation of toxicity for several compounds, whereas for others the toxicity may be unchanged or even reduced (Strubelt, 1978). Because participation of cytochrome-P450 is important in the activation of the tested pregenotoxic compounds, one would expect a change in the mutagenic potential of the tested compounds after metabolic activation with hepatocytes from ethanol pretreated rats compared

with hepatocytes from controls. Ethanol pretreatment resulted in an increased mutagenicity of BZ, INH, CP, DMN and DEN. However, the mutagenicity of 2-AF and 2-AAF was not influenced (Figure 1). Ethanol pretreatment significantly enhanced (by 45%) the N-acetylation capacity of the isolated hepatocytes (Table I). Olsen (1978 and 1982) reported an ethanol induced increase in the rate of sulfadimidine and procainamide acetylation in man. They also found an ethanol induced increase of about 30% in the rate of sulfadimidine acetylation in suspensions of isolated rat hepatocytes (Olsen, 1978). Whether our observed increase in N-acetylation is the result of an increase in the amount of N-acetyltransferase, or due to an increase in acetyl-CoA, the cofactor for the N-acetylation reaction, as suggested by Olsen (1978), is yet unclear.

The conversion of BZ, 2-AF and 2-AAF to metabolites capable of interaction with cellular macromolecules is important with respect to their genotoxic properties. The metabolic activation of arylamines is a complex process, which is believed to proceed via N-hydroxylation and esterification. These compounds can also undergo N-acetylation in a number of species, including man. The balance between acetylation and deacetylation plays an essential role in determining susceptibility to arylamine genotoxicity (Neis, 1984 and 1985). Therefore, one might expect that the observed increase in N-acetylation will influence the activation of BZ, 2-AF and 2-AAF into mutagenic products. For monofunctional arylamines like 2-AF, 4-aminobiphenyl and 2-aminoanthracene the N-acetylation reaction seems to reduce the mutagenic potency (Connor, 1983; Neis, 1984). Conner et al. (1983) showed that the mutagenic potency of 2-AAF was significantly lower than the corresponding 2-AF. In contrast, for the bifunctional arylamine BZ, the N-acetylation is an essential step in the metabolic activation of this compound into genotoxic species (Martin, 1982; Neis, 1984).

Therefore, we suggest that the increase in N-acetylation will enhance the mutagenicity of BZ but will decrease the mutagenicity of 2-AF and 2-AAF. The observed increase in BZ mutagenicity (Figure 1a) is compatible with the increased N-acetylation rate

and cytochrome-P450 content. The unchanged mutagenicity of 2-AF and 2-AAF (Figure 1a) may be explained by the fact that opposite effects on mutagenicity, due to an increase in N-acetylation (deactivation) and cytochrome-P450 dependent deactivation reactions on the one hand and an increase in cytochrome-P450 dependent activation on the other, compensate for each other.

DMN has also been used as a model compound in other studies concerning the influence of ethanol on the bioactivation of pregenotoxic agents. In vitro studies with subcellular liver fractions showed that DMN is more effectively activated to a mutagen with preparations from the livers of ethanol pretreated rats than from controls (Garro, 1981; Smith, 1984). The alpha-carbon hydroxylation, which is mediated by cytochrome-P450 mono-oxygenases is a crucial step in the bioactivation of N-nitrosamines to ultimate genotoxic species (Lai, 1980). Experiments with DMN have shown that the activity of the microsomal cytochrome-P450 dependent DMN-demethylase which catalyzes the initial alpha-hydroxylation of DMN is increased by ethanol pretreatment (Garro, 1981; Schnellmann, 1984; Schwartz, 1980; Smith, 1984). Maling et al. (1975) reported an enhanced hepatotoxicity of DMN after pretreatment of rats with ethanol. Olson et al. (1984) found a potentiation of unscheduled DNA synthesis, following exposure to DMN, with isolated hepatocytes from ethanol pretreated rats compared with hepatocytes from controls. Our observation that the mutagenicity of DMN and DEN is higher with hepatocytes from ethanol pretreated rats than with hepatocytes from control rats (Figure 1b), is compatible with these findings. However, Griciute et al. (1981) and Teschke et al. (1983) found that ethanol failed to change the total number of tumors significantly in C57BL mice and rats, respectively.

The antitubercular drug INH is mainly acetylated to acetyl-INH which in turn forms acetylhydrazine upon metabolic hydrolysis. The liberated acetylhydrazine can be converted by cytochrome-P450 dependent metabolism into potent acylating agents, capable of reacting with cellular macromolecules (Timbrell, 1980). The importance of the N-acetylation reaction in the bioactivation of INH was also underlined by other investigators (Gatehouse, 1984;

Mitchell, 1975). Mitchell et al. (1975) showed an increased incidence of INH-hepatitis on rapid acetylators. Gatehouse et al. (1984) reported that the primary step in the metabolic activation of INH to mutagens, in the fluctuation assay appeared to be mediated by an N-acetyltransferase. In a study on rats (Thomas, 1977), ethanol increased the excretion of N-acetyl-INH. We therefore suggest that the increase in N-acetylation and cytochrome-P450 content in the hepatocytes of the ethanol pretreated rats result in a more effective activation of INH by these cells, thereby explaining the observed increase in mutagenicity of INH after ethanol pretreatment (Figure 1c).
Ethanol pretreatment resulted in an increase in the mutagenic activation of the cytostatic CP (Figure 1c). This may be due to the ethanol induced cytochrome-P450 dependent activation of CP.
These results support the hypothesis that induction of the hepatic drug metabolizing system may be responsible for the ethanol induced increase in susceptibility to certain genotoxic agents.

ACKNOWLEDGEMENT

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Chapter XI

DISAPPEARANCE OF FREE SH-GROUPS IN HEMOGLOBIN OF MAN, RAT AND RABBIT AFTER EXPOSURE TO ALKYLATING AGENTS IN VITRO

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SUMMARY

The effect of alkylating agents on the content of free SH-groups in human, rat and rabbit hemoglobin was examined. Treatment of blood in vitro with iodoacetamide, styrene oxide and methylmethanesulfonate resulted in a dose-dependent decrease of free SH-groups in hemoglobin. The free SH-groups of rat hemoglobin were more susceptible to the alkylating agents tested than those of human and rabbit hemoglobin. The method is based on the isolation of hemoglobin and a subsequent measurement of the free SH-content of the hemoglobin by means of a spectrophotometric procedure. This procedure may be useful to examine differences in susceptibility of free SH-groups in hemoglobin, e.g. of different animal species, and on the other hand, to study the potencies of different electrophilic agents to alkylate these nucleophilic centers.

INTRODUCTION

Many compounds, including most chemical carcinogens, derive their injurious properties from their conversion into electrophilic metabolites, that can react with nucleophilic groups of cellular macromolecules. Other potential carcinogens are reactive electrophiles per se. These are alkylating, arylating or acylating agents, present in cigarette smoke, widely used as industrially significant reagents, but also as cytostatic drugs in chemotherapy of cancer.

Ehrenberg and coworkers observed alkylated products of cysteine and histidine in hemoglobin after administration to mice of radiolabelled ethylene oxide (Ehrenberg, 1974), methylmethanesulfonate (Segerbäck, 1978), dimethylnitrosamine (Osterman-Golkar, 1976), and vinyl chloride (Osterman-Golkar, 1977). Furthermore, after administration to mice of radiolabelled methylmethanesulfonate, the amount of

S-methylcysteine and N-3-methylhistidine decreased linearly with time at the same rate as erythrocyte degradation (Segerbäck, 1978). Pereira et al. (1981) also showed that a broad spectrum of chemical classes of carcinogens covalently bind to hemoglobin after administration of these compounds to rats.

Between animal species great differences are known in the toxic effects of electrophilic agents. Interspecies differences may also be expected in the extent of the alkylation of nucleophilic groups, like free SH-groups in hemoglobin.

In the present investigation we used a simple, non-selective method to examine the covalent binding of electrophiles to free SH-groups in hemoglobin of man, rat and rabbit. The method is based on the disappearance of free SH-groups in hemoglobin after treatment of blood with iodoacetamide, styrene oxide and methylmethanesulfonate.

MATERIALS AND METHODS

Chemicals

Iodoacetamide and 4,4'-dithiopyridine were purchased from Janssen Chimica (Beerse, Belgium). Methylmethanesulfonate was obtained from Aldrich Chemical Co. Inc. (Milwaukee, USA). Styrene oxide was obtained from J.T. Baker Chemicals (Deventer, The Netherlands). All other chemicals used were of high purity.

Blood samples

Animal blood was collected under ether anesthesia by cardiopuncture with a heparinized syringe. Adult male Wistar rats and adult male New Zealand White rabbits were used. Samples of fresh heparinized blood of healthy male volunteers were obtained from a blood bank.

In vitro incubation of whole blood with alkylating agents

In a shaking waterbath (210 rpm) closed sterile vials (i.d. 24 mm, height 55 mm), each containing 3 ml of sterile heparinized blood and 100 ul of a sterile solution (in saline or DMSO) of the compound under test, were incubated at 37 oC for 5 h.

Isolation and purification of human and rabbit hemoglobin

After incubation, hemoglobin was isolated and purified as follows. The blood samples were suspended in 2 volumes of ice-cold PBS (phosphate-buffered saline (pH 8.0); 10mM Na-phosphate/150 mM NaCl) and centrifuged at 2300 xg at 4 oC for 5 min. The supernatant and the buffy coat were removed by careful suction, and the erythrocytes were resuspended in 5 volumes of ice-cold PBS. After mixing, the samples were centrifuged again at 2300 xg at 4 oC for 5 min. The supernatant was removed by careful suction, and a few erythrocytes were sacrificed to remove any remaining buffy layer. This washing procedure was repeated twice. The erythrocytes were then lysed carefully in 2 volumes of ice-cold distilled water on ice for 15 min. Next, the ionic strength was brought to the original value by the addition of a 50 mM Na-phosphatebuffer (pH 8.0), containing 750 mM NaCl. The samples were then centrifuged at 10000 xg at 4 oC for 20 min to remove cells and cellular debris. The supernatant was dialysed against PBS overnight (for 17 h; after 5 h the PBS was exchanged).

Isolation and purification of rat hemoglobin

The isolation and purification procedure was the same as for human and rabbit hemoglobin except for the use of PBS that was replaced by a 5 mM Tris-glycine-HCl buffer (pH 8.8), containing 150 mM NaCl. The latter buffer was applied to prevent the crystallization of rat hemoglobin (Stein, 1971; Brunori, 1982;

Condò, 1981)

Determination of hemoglobin content in the hemoglobin samples

The hemoglobin assay is based on the hemiglobincyanide-procedure of van Kampen en Zijlstra (1961). Fifty microlitres of the hemoglobin sample was mixed with 2.5 ml of a buffer/reagent solution, made up of 1 mM KH_2PO_4, 0.75 mM KCN and 0.6 mM $K_3Fe(CN)_6$, in brown-glass stoppered test tubes. The absorbance at 540 nm was read against water after 30 min of incubation in a waterbath at 25 oC. Longer delays did not affect the absorbance. Hemoglobin standards were used to estimate the molar extinction coefficient of the hemiglobincyanide formed, and showed to be $10.5 \times 10^3\ M^{-1}cm^{-1}$.

Determination of sulfhydryl groups in hemoglobin

Sulfhydryl groups were determined according to the procedure of Grassetti and Murray (1967), with slight modifications. The procedure is based on the reaction of 4,4'-dithiopyridine (4-PDS) with sulfhydryl groups, giving the corresponding 4-thiopyridone (4-TP). The ultraviolet absorption of the 4-TP formed, which is proportional to the sulfhydryl content of the sample, was measured at 324 nm.

A stock solution of 1 mM 4-PDS was prepared by dissolving 22 mg 4-PDS in 10 ml distilled water. Because of the relative insolubility of the uncharged 4-PDS in water, it was necessary to stir the solution at room temperature (protected from light) for approximately 2 h. The resulting solution proved to be stable for up to 2 months when stored at 4 oC.

One hundred microlitres of each purified hemoglobin sample was diluted with 15 ml phosphate/EDTA-buffer (0.0282 M KH_2PO_4/ 0.0718M Na_2HPO_4-buffer (pH 7.15), containing 1 mM EDTA). The diluted hemoglobin samples contained about 350 ug Hb/ml. Aliquots of 3 ml were mixed with 200 ul of a 1 mM 4-PDS solution

in brown-glass stoppered test tubes. The absorbance at 324 nm was read against water after 40 min of incubation in a waterbath at 25 oC. Longer delays did not affect the absorbance. An appropriate hemoglobin blank (made up of 3 ml of the hemoglobin solution and 200 ul aqua pure) and a 4-PDS blank (made up of 3 ml phosphate/EDTA-buffer and 200 ul of the 1 mM 4-PDS solution), were carried through incubation. All the incubations were done in duplicate. The sulfhydryl content of the hemoglobin samples was calculated from the corrected extinction value and the molar extinction coefficient of the 4-TP formed. Standard solutions of cysteine were used in the estimation of the molar extinction coefficient of the 4-TP, and showed to be 20.5×10^3 $M^{-1}cm^{-1}$.

Apparatus

Spectra were taken on a Varian Cary 118 spectrophotometer. Absorption measurements were made on a Pye Unicam SP 1750 spectrophotometer equipped with a flow-cell.

RESULTS

Calibration of the spectrophotometric Hb assay

It was ascertained whether measurement of absorbance at 324 nm of Hb samples prepared from human, rat or rabbit blood can be used for the assay of Hb concentration in these samples. Therefore, samples with known Hb concentrations (accurately determined with the hemiglobincyanide-method as described in the "Materials and Methods" section) were diluted with phosphate/EDTA-buffer (pH 7.15); subsequently the absorbance at 324 nm was measured against water. Figure 1 shows that the absorbance at 324 nm of human, rat and rabbit hemoglobin is a linear function of the Hb concentration. The absorption spectrum (250-650 nm) of the hemoglobin of man, rat and rabbit showed to be identical.

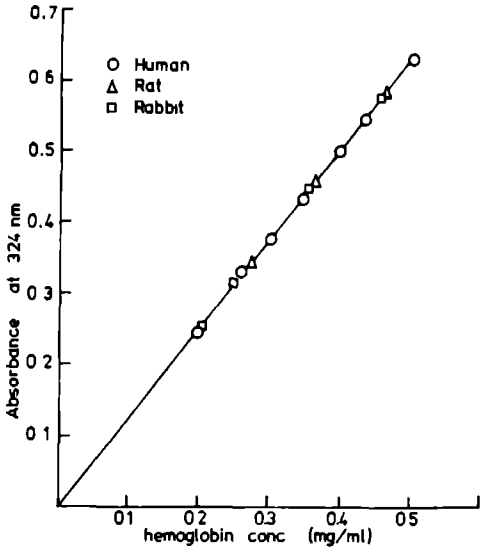

Fig. 1. Calibration of the absorbance at 324 nm of Hb samples prepared from human, rat and rabbit blood. Each point represents the average of three determinations (S.D. is less than 0.5%).

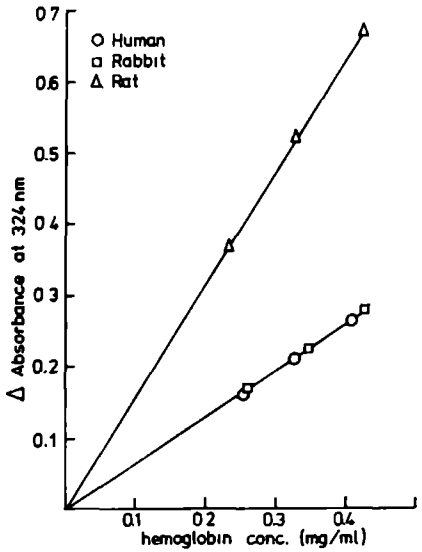

Fig. 2. Effect of the Hb concentration on the titration of SH-groups in hemoglobin of man, rat and rabbit, with 4-PDS. Each point represents the average of three determinations (S.D. is less than 0.5%).

Treatment of Hb with iodoacetamide, styrene oxide or methylmethanesulfonate did not alter these spectra. From the data of Figure 1 it can be calculated that $E^{Hb4}_{M324} = 80.6 \times 10^3\ M^{-1}cm^{-1}$. This extinction coefficient was used in further experiments in the assay of the Hb concentration in the Hb samples. The advantage of this method is that the samples that serve as Hb blanks in the SH assay (see "Materials and Methods"), can be used for the determination of the Hb concentration at the same time.

Free SH-groups in untreated hemoglobin of man, rat and rabbit

The concentration of free SH-groups was derived from the concentration of 4-TP that is released during the reaction of 4-PDS with the free SH-groups of hemoglobin. 4-TP concentrations were determined spectrophotometrically at 324 nm. The absorbances measured were corrected for the contribution of 4-PDS and Hb using a blank 4-PDS solution and the blank Hb samples (without 4-PDS). The absorbances at 324 nm due to the blank Hb samples were also used to calculate Hb concentrations as mentioned above. Figure 2 shows that the absorbance at 324 nm of the 4-TP, formed during the reaction of hemoglobin with 4-PDS, is proportional to the Hb concentration of the Hb samples tested. It can be calculated from these data that human as well as rabbit hemoglobin contain 2 free SH-groups and rat hamoglobin contains 5 free SH-groups, by using a $E^{4\text{-}TP}_{M324} = 20.5 \times 10^3\ M^{-1}cm^{-1}$.

Disappearance of free SH-groups after alkylation

The disappearance of free SH-groups in hemoglobin of man, rat and rabbit, after in vitro treatment of whole blood with iodoacetamide, styrene oxide and methylmethanesulfonate is illustrated in Figure 3.
Figure 3a shows that the reaction of iodoacetamide with free

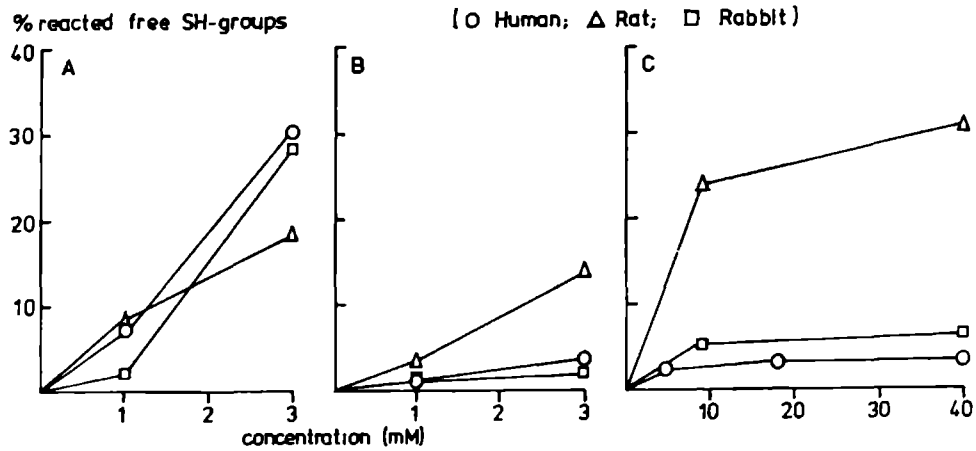

Fig. 3. Reaction of free SH-groups in hemoglobin of man, rat and rabbit after treatment of whole blood in-vitro with iodoacetamide (A), styrene oxide (B) and methylmethanesulfonate (C). Each point represents the average of two determinations (S.D. is less than 0.5%).

SH-groups of human, rat and rabbit hemoglobin is concentration-dependent. Relatively high responses were obtained with the hemoglobin of all three species.

Styrene oxide reacted more effectively with the free SH-groups in rat hemoglobin than human or rabbit hemoglobin. The response of human and rabbit hemoglobin to this compound appeared to be relatively low.

As presented in Figure 3c a similar phenomenon was observed with methylmethanesulfonate which reacted with the free SH-groups in hemoglobin of all three species. Rat hemoglobin, however, was most responsive.

It was further studied to ascertain whether the low reactivity of the free SH-groups in human hemoglobin to methylmethanesulfonate might be due to a failure of this compound to penetrate the erythrocyte membrane or was the consequence of competing reactions with other nucleophilic groups in the blood. Therefore, methylmethanesulfonate was also incubated with purified human hemoglobin instead of whole blood. After this incubation, however, subsequent analysis again revealed a very low response, comparable to that obtained with whole blood.

Human and rabbit blood each contain one major hemoglobin (Drysdale, 1971; Dayhoff, 1978) whereas rat blood has 4 major- and 2 minor hemoglobins (Stein, 1971; Brunori, 1982; Dayhoff, 1978). Friedman (1973)and Dayhoff (1978) reported 2 free SH-groups/hemoglobin tetramer in human and rabbit blood. Rat blood, however, contains six different hemoglobins, with 4,5 or 6 free SH-groups (Condò, 1981). In human and rabbit hemoglobin only the cysteine, contains a reactive SH-group. In rat hemoglobin, besides the cysteine, also the cysteine contains a reactive SH-group (Condò, 1981); other cycteines may also be reactive. The cysteine in rat hemoglobin is located at the outside of the hemoglobin molecule and will therefore present a more prefered SH-group for alkylation than the cysteine, which is located inside the hemoglobin molecule.
In accordance with this, the present results show 2 SH-groups/human and per rabbit hemoglobin tetramer and 5 SH-groups/rat hemoglobin tetramer reacting with 4-PDS (Figure 2). The results shown in Figure 3 demonstrate that exposure to increasing concentrations of the alkylating agents results in an increase in the percentage of free SH-groups that have reacted. The dose dependency of the response suggests that the loss of free SH-groups is the consequence of an interaction between these groups and the electrophilic agent. It is further illustrated in this figure that there are differences in the potency of electrophilic agents to react with free SH-groups in hemoglobin. Alkylation of free SH-groups in hemoglobin may depend on the concentration and reactivity of the electrophile and, in addition, on the presence, nucleophility and accessibility of the SH-groups. The latter is mainly determined by steric conditions. Variations in the number and position of the free SH-groups may be responsible for the observed differences in susceptibility to alkylation between rats on one hand, and humans and rabbits on the other (Figure 3). For methylmethanesulfonate, only minor alkylation of free SH-groups in human hemoglobin was found, both

after incubation with whole blood and with purified hemoglobin. So it seems unlikely that there is a penetration barrier for this alkylating agent. Possibly, the low reactivity of methylmethanesulfonate towards the free SH-groups is due to competing reactions with other amino acids in the hemoglobin, e.g. histidine.

Detection of alkylated hemoglobin can be considered as an important tool in monitoring human exposure to electrophilic compounds. Therefore, several investigators have developed selective methods, like GC-MS analysis of modified amino acids of hemoglobin, which have proved to be very sensitive (Ehrenberg, 1977; Calleman, 1978; Farmer, 1980 and 1982; Bailey, 1981). In this respect it remains doubtful whether the indirect assay of the alkylation of SH-groups, as described in this paper, because of its low sensitivity, is suitable for the detection of human exposures to low concentrations of electrophiles. Nevertheless, the simplicity of the procedure makes it suitable for routine uses, for instance to study the potency of various electrophilic chemicals to react with free SH-groups, and on the other hand, to examine strain- or species-dependent differences in susceptibility of hemoglobins to the action of alkylating agents.

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In-vitro/in-vivo differences

It is questionable whether the metabolic activation of pregenotoxic chemicals by S9 fractions is representative for the biotransformation in-vivo. Several investigators have pointed out already that there are great differences in metabolite profiles, DNA-adduct profiles and mutagenicity responses, between in-vitro assays using metabolic activation with S9 preparations and those applying intact cells, showing that intact cells reflect the in-vivo situation more properly (Bartsch, 1983; Bos, 1982; Bridges, 1980; Brouns, 1979; Gould, 1983; Selkirk, 1977). This consideration is affirmed by several results of the present thesis. In chapter III the mutagenicity of two structurally related carcinogenic arylamines, benzidine and 4-aminobiphenyl, after metabolic activation by S9 liver fractions or isolated intact hepatocytes, was investigated. It appeared that with intact hepatocytes the mutagenic responses more closely approximated those obtained with in-vivo bioactivation in animals. The different results between experiments with S9 and intact hepatocytes were found to be due to the lack of N-acetylating capacity of the S9. It appeared that no appropriate cofactor levels, i.e. acetyl-CoA were present in the subcellular S9 preparation of the liver. Consequently, activation reactions, e.g. N-acetylation in the case of benzidine, and deactivation reactions, e.g. N-acetylation in the case of 4-aminobiphenyl, are deficient in S9. This might result in unreliable predictions, in case the outcome of S9-dependent assays should be used in risk estimations. This phenomenon is again underlined by the results of studies on the mutagenicity of N-acetylbenzidine, N,N'-diacetylbenzidine and isoniazid (chapter IV and IX). Even for genotoxic compounds of other chemical classes it appeared that as well for rat as man the genotoxic activation by S9 greatly differed from that of the intact hepatocytes (chapter VII).

It is remarkable that, benzidine, dimethylnitrosamine, diethylnitrosamine and isoniazid were weakly- or non-mutagenic, when activated with rat or human liver S9, but highly mutagenic in the presence of intact hepatocytes (chapters III, VII, IX). The latter is well compatible with the high activity of these compounds in vivo. In contrast, B(a)P appeared highly mutagenic in the presence of rat liver S9, whereas with intact hepatocytes it was far less mutagenic. The latter is in accordance with the observation that the rat liver is not a target for the carcinogenic action of B(a)P in vivo. The relatively high activating potential of rat liver towards B(a)P, as can be concluded from the relatively high rate of metabolism of B(a)P (chapter VII, figure 2) will be present in S9 as well as in intact hepatocytes, however the known high activity of the liver glutathione conjugation reaction (deactivation) will only be present in intact hepatocytes (Brouns, 1979; Jernström, 1984).
From the studies mentioned above it can be concluded that in in-vitro genotoxicity studies intact hepatocytes are a better model for the in-vivo bioactivation than the generally used S9 liver fractions. Using subcellular preparations critical toxification as well as detoxification reactions may easily be overlooked. This explains, at least in part the many discrepancies between in-vivo and in-vitro (geno)toxicity data. The present results suggest that the use of isolated, intact cells can basically contribute to bridge the in-vitro/in-vivo gap.

Interspecies differences

Qualitative as well as quantitative differences in metabolism and susceptibility to genotoxic chemicals between species are well known (Clayson, 1983; Conney, 1974; ECETOC, 1982; Juchau, 1983; Kato, 1979; Motulsky, 1982; Streissinger, 1983). To have more insight in the underlying mechanisms of these variations several fundamental in-vitro studies were undertaken.
In the chapters III-VI variations in genotoxicity of some

arylamines and arylamides were studied in relation to the particular potencies of bioactivation in isolated hepatocytes of several animal species and man. These studies revealed substantial quantitative as well as qualitative interspecies variations in the genotoxicity profiles. An obvious example was the relatively low activity of these compounds in the presence of hepatocytes from the guinea pig, a species reported to be resistant to the carcinogenic action of arylamines and arylamides, like 2-AF and 2-AAF. These studies showed that the balance between acetylation and deacetylation is one of the determinant factors in the ultimate genotoxicity of arylamines and arylamides in several species including man. On the other hand, interspecies differences in the N-hydroxylation, probably due to interspecies differences in substrate specificity of the cytochrome-P450 system towards these compounds, which are mainly determined by interspecies differences in isoenzymes composition, also play a crucial role in the ultimate genotoxicity.

In chapter VII a more extensive evaluation of differences in metabolic activation between rat- (probably the most frequently used animal species in genotoxicity studies) and human liver preparations was obtained by studying the mutagenicity of compounds from different chemical classes, known to be activated via different metabolic routes. A striking observation was the difference in mutagenicity of B(a)P in the standard Ames-assay. The mutagenicity of B(a)P was high, when activated by rat liver S9 and low in the presence of human liver S9. This difference could be attributed to differences in B(a)P metabolism, namely to a far smaller capacity of the activating pathways in the human liver S9 compared with the rat liver S9 (chapter VII).

Even with hepatocytes of a monkey (Macaca fascicularis) -a primate species evolutionary more closely related to man-, great qualitative and quantitative dissimilarities in genotoxic activation were seen when compared with human hepatocytes (chapter VIII). A striking difference was the high mutagenicity of B(a)P in the presence of the monkey hepatocytes compared with the low activity in the presence of human hepatocytes.

In chapter IX the genotoxicity was studied of the widely used

antituberculous drug isoniazid. From clinical and animal research data it is known that this compound can produce, by electrophilic interactions, several types of liver injury (e.g. necrosis) in man. The rat liver, however, was resistant to it. Our results revealed that as far as the genotoxic activity of this drug is concerned, hepatocytes of certain human individuals are far more effective in bioactivating isoniazid to genotoxic products than rat hepatocytes. It was concluded that in studies of the genotoxic and/or hepatotoxic action of this drug the rat is not a proper model for man.

Interindividual differences

Even within one species, marked differences in the metabolic activation of chemicals to genotoxic products can be expected. This problem was approached by the studies described in chapter VII and IX. Isolated hepatocytes derived from different persons were examined for their ability to bioactivate several chemicals to mutagenic products. Considerable interindividual differences in activating potency were found, ranging to about 100-fold, depending on the chemical tested. Harris et al. (1984) reported that the quantity of carcinogen DNA-adducts formed in cultured human epithelial tissue explants, after exposure to different chemical carcinogens, varied to 150-fold among cultured tissues from different people. These variations are not unexpected, if one takes into consideration the known great interindividual differences in the metabolism of xenobiotic chemicals (Dybing, 1979; Harris, 1984; Moore, 1984; Motulsky, 1982; Nebert, 1983; Omenn, 1984) and the close relationship between metabolism and genotoxicity (e.g. chapter III-X). Most obvious were the great interindividual differences in metabolic activation of benzidine and isoniazid. It is noteworthy that the hepatocytes of individuals capable of more effectively activating benzidine were also more potent in activating isoniazid. These results correspond with earlier findings that liver N-acetylation of

isoniazid as well as benzidine are genetically controlled, dividing people in "slow and rapid acetylators". In chapter IX supporting evidence is presented for the hypothesis that rapid acetylator humans metabolize isoniazid more effectively to genotoxic products. To backbone this hypothesis and to find out whether this hypothesis also holds for benzidine, further investigations with human liver preparations have to be done. McQueen et al. (1983) have shown that benzidine was far more effectively activated to DNA-damaging products by hepatocytes of rapid acetylator rabbits than slow acetylator rabbits, thereby giving support to the above mentioned hypothesis.
In addition to the genetic predisposition, the degree of exposure to environmental modifiers, in particular enzymes inducers, can also be held responsible for the interindividual variations in (geno)toxic activation. It was suggested, therefore, that the fact that the hepatocytes derived from a heavy drinker showed a relative high capacity to activate certain chemicals to mutagens, was due to ethanol induction (chapter V).
In chapter X this effect of ethanol was studied in rats. Prolonged ethanol feeding to rats influenced the capacity of isolated hepatocytes to activate several chemicals to mutagenic products. This study revealed that ethanol pretreatment led to several changes in the metabolic fate of the chemicals tested. These changes were held responsible for the observed alterations in genotoxicity.

Target susceptibility

Due to the large amount of data about the biotransformation of pretoxic compounds, there may be the risk of underestimating the significance of non-metabolic factors, that determine the rate of electrophilic attack, e.g. the sensitivity of the target molecules. In the framework of the present thesis minor attention was given to this problem.
Chapter XI was devoted to a study of the alkylation of free

SH-groups in hemoglobin of different species including man by direct alkylating agents. In other words, the interaction was measured of nucleophilic targets with agents that do not need metabolic activation. Significant interspecies differences were observed in the susceptibility of the free SH-groups towards alkylation. It was observed that free SH-groups in rat hemoglobin were far more sensitive than those of rabbit and man. This variation is probably due to the existence of "hot" free SH-groups in the rat hemoglobin.
Several reports have also indicated the existence of so-called "hot-spots" in the DNA, showing these parts of the DNA to be far more susceptible to DNA-damage than other parts. It is likely to assume that these hot-spots are subject to species-, sex- or even interindividual differences resulting in variations in susceptibility to genotoxic compounds.

Concluding remarks

To ensure a safe utilization of man-made and other chemicals in our society a tremendous amount of compounds has to be tested for their potential (geno)toxicity. Until recently these studies were performed for the greater part with intact animals. Animal-bioassays, however, have evolved to be highly complex, extremely expensive and time consuming experiments, involving large numbers of animals. Therefore, the need has developed for more ethical and economical methodologies. This led to the development of a great diversity of short-term in-vitro screening assays. However, there is much concern about the predictive value of results obtained from in-vitro (geno)toxicity studies.
Several reports, including the present thesis have demonstrated, however, that the gap between in-vitro and in-vivo studies might be bridged partly by the use of intact cell-systems, reflecting some typical features of total organisms. Thereby, reinforcing the value of in-vitro investigations for (geno)toxicity

evaluation.

Extrapolations to humans from observed effects in animals in-vivo are seriously hampered by strain- and species differences in susceptibility among experimental animals. This is mostly caused by variations in biotransformation. The availability of human in-vivo genotoxicity data is limited (fortunately). Moreover, cause and effect relationships are difficult to establish in humans. There is no simple approach for this complicated problem. Appropriate in-vitro studies using human target tissue or cells may bring relief. As is demonstrated in the present thesis, isolated hepatocytes can be used for metabolic activation and/or as target cells. Extension of the use of such experiments to studies in which isolated human liver cells are co-incubated or co-cultivated with human cells of other organs or tissues (e.g. lung, kidney, skin) will even strengthen this approach. Recently, Strom et al. (1983) have used isolated human hepatocytes as metabolic entity together with human fibroblasts as target cells, to study the genotoxicity of several chemicals. In terms of risk identification, such findings are still very preliminary and many further studies are needed. However, in view of a serious attempt to replace in-vivo animal experiments by in-vitro research, it is a challenging question whether, by the use of human in-vitro cell-systems, the two-step extrapolation (from animal in-vitro to animal in-vivo and from animal in-vivo to man in-vivo) can be passed over (Figure 1: step 3, instead of step 1 + 2) or, on the other hand, whether human cell-systems are valuable but additional tools only. The results of the present thesis are promising in both aspects.

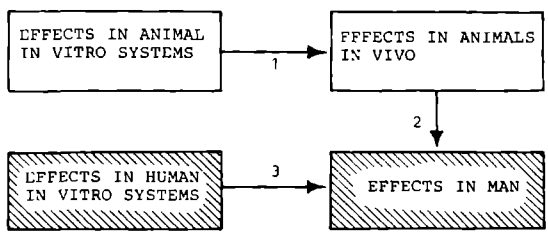

Fig. 1. Extrapolation of toxicological data from various experimental systems

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Many body-foreign compounds are capable, mostly after metabolic activation, to evoke in the organism a diversity of genotoxic effects. Great inter- and intraspecies differences in susceptibility to potentially genotoxic chemicals are known. The underlying mechanisms of these variations are not fully understood.

The present thesis was primarily directed to obtain more insight in these variations. Attention was mainly focussed on differences in metabolic activation as a cause of dissimilaries in effects. In-vitro studies were performed with some genotoxic model-compounds using isolated hepatocytes of different species, as metabolic entity and/or as the target cells. Apart from intact hepatocytes also a subcellular liver fraction, i.e. the 9000 xg supernatant (S9), was used as a metabolic system. The species were rat, hamster, guinea pig, dog, monkey and man.

Great discrepancies in metabolic activation of genotoxic chemicals between S9 liver fractions and intact hepatocytes were observed, showing that intact hepatocytes reflected more properly the bioactivation in-vivo. Benzidine and dimethylnitrosamine, for instance, were almost non-mutagenic when activated by rat and human S9 liver fractions, whereas in the presence of intact hepatocytes of either species high mutagenicity values were found. In addition, qualitative differences were observed. Benzidine was far more mutagenic than 4-aminobiphenyl when activated with intact rat hepatocytes, however in the presence of S9 liver fractions the reverse was found.

The results obtained suggest that a great part of the existence of species-, strain-, sex- and other interindividual differences in susceptibility to genotoxic compounds may be attributed to differences in activation and deactivation processes that are influenced by genetic and/or environmental factors. Ethanol-feeding to rats, for instance, influenced the capacity of isolated hepatocytes to activate benzidine, dimethylnitrosamine,

cyclophosphamide and isoniazid, to mutagenic products by changing the metabolic fate of these compounds. Isoniazid, a widely used antituberculous drug, appeared to be non-genotoxic when activated by rat hepatocytes, however, in the presence of human hepatocytes of certain subjects it was found to be highly genotoxic. Indications were obtained that the latter phenomenon is associated with the genetic predisposition of the human subject for high or low capacity of hepatic N-acetylation.
Furthermore, interspecies differences were found in the susceptibility of target centers in certain macromolecules, i.e. the free SH-groups in hemoglobin, towards electrophilic attack.
In defining factors underlying inter- and intraspecies specificity of potentially genotoxic chemicals, in-vitro systems of intact cells have been shown a valuable tool. The use of human (liver)cells in in-vitro studies offers the oppertunity to obtain by experiments genotoxicity data concerning man, the species of primary interest.

Veel lichaamsvreemde stoffen zijn in staat om in het organisme, meestal na metabole activering, meerdere genotoxische effecten te bewerkstelligen. Aanzienlijke inter- en intraspecies verschillen in de gevoeligheid voor potentieel genotoxische chemicaliën zijn bekend. De mechanismen die hieraan ten grondslag liggen zijn veelal nog onduidelijk.
Dit proefschrift is er primair op gericht om meer inzicht te verkrijgen in deze verschillen, met de nadruk op de metabole activering als mogelijke oorzaak. Bij in-vitro studies, uitgevoerd met enkele genotoxische modelstoffen, werd gebruik gemaakt van geïsoleerde hepatocyten van verschillende species. De hepatocyten fungeerden als metabool activerend systeem en/of als de "target" cellen. Daarnaast werd ook een subcellulaire leverfractie, namelijk 9000 xg supernatant (S9), gebruikt als metaboliserend systeem. De volgende species werden gebruikt, rat, cavia, hamster, hond, aap en de mens.
Grote discrepanties werden gevonden in metabole activering van genotoxische stoffen tussen S9 leverfracties enerzijds en intacte hepatocyten anderzijds. Hieruit bleek dat de intacte hepatocyten op een meer betrouwbare wijze de in-vivo bioactivering weergeven. Bijvoorbeeld, benzidine en dimethylnitrosamine bijvoorbeeld, waren nagenoeg niet-mutageen na activering met ratte- en humane S9 leverfracties, terwijl in aanwezigheid van intacte hepatocyten van elk van beider species een hoge mutageniteit werd gevonden. Tevens werden kwalitatieve verschillen gevonden. Benzidine bleek veel mutagener dan 4-aminobiphenyl na metabole activering met intacte rattehepatocyten, terwijl in aanwezigheid van S9 leverfracties het omgekeerde werd gevonden.
De verkregen resultaten wijzen erop dat een groot deel van de species-, stam-, geslachts- en overige interindividuele verschillen in gevoeligheid voor genotoxische stoffen kunnen worden toegeschreven aan verschillen in activerings- en deactiverings processen. Deze processen worden beinvloed door

genetische en/of omgevingsfactoren. Enkele voorbeelden mogen dit verduidelijken. Toediening van ethanol aan ratten via het drinkwater had grote invloed op de capaciteit van de geïsoleerde hepatocyten om benzidine, dimethylnitrosamine, cyclofosfamide en isoniazide, om te zetten in mutagene producten. Dit effect was het gevolg van een verandering van het metabolisme van deze stoffen. Isoniazide, een veel gebruikt geneesmiddel tegen tuberculose, bleek niet genotoxisch te zijn na activering met rattehepatocyten, terwijl het na metabole activering met humane hepatocyten afkomstig van sommige personen, sterk genotoxisch bleek te zijn. Er werden aanwijzingen gevonden dat dit laatst genoemde fenomeen verband houdt met een genetische predispositie voor een hoge of lage N-acetyleringscapaciteit van de lever ("snelle en langzame acetyleerders").

Op het gebied van de gevoeligheid van bepaalde "target-centra" van macromoleculen voor electrofiele agentia werden eveneens interspecies verschillen gevonden. Dit betreft met name de alkyleerbaarheid van vrije SH-groepen in hemoglobine.

Uit de resultaten van dit proefschrift blijkt, dat in-vitro systemen waarin intacte cellen worden gebruikt een waardevol hulpmiddel zijn bij het bestuderen van factoren die ten grondslag liggen aan inter- en intraspecies specificiteit van potentieel genotoxische chemicaliën. Het gebruik van humane (lever)cellen bij in-vitro studies biedt de mogelijkheid om via experimenten genotoxische gegevens te verkrijgen die betrekking hebben op de mens, de species waarin we primair geïnteresseerd zijn.

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John M. Neis werd geboren te Sittard op 4 september 1958. In 1976 behaalde hij het diploma Atheneum B aan het Bisschoppelijk College te Sittard. Daarna studeerde hij chemie aan de Katholieke Universiteit te Nijmegen, waarna hij op 24 september 1979 het kandidaatsexamen S2 aflegde. Het doctoraaldiploma met als hoofdvak Farmacochemie (Toxicologie) met als bijvakken Chemische Cytologie en Chemische Microbiologie en een caput Organische Chemie (synthese) behaalde hij op 27 december 1982. Van 1 november 1982 tot juli 1983 was hij in dienst van de Katholieke Universiteit Nijmegen, als tijdelijk wetenschappelijk medewerker bij de werkgroep Toxicologie op basis van een subsidie van het Directoraat-Generaal van de Arbeid. Hier participeerde hij binnen het project: "biologishe-monitoring industriële carcinogenen". Vanaf 1 juli 1983 is hij in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO) en verbonden als wetenschappelijk medewerker bij de werkgroep Toxicologie te Nijmegen aan het project: "interspecies-verschillen in toxische effecten van potentieel alkylerende stoffen". Momenteel is hij als toxicoloog werkzaam bij de afdeling Arbeidshygiëne en Toxicologie van de Centrale Veiligheids- en Milieudienst van DSM.

STELLINGEN

behorende bij het proefschrift

VARIATION IN GENOTOXICITY DUE TO DIFFERENCES IN METABOLIC ACTIVATION

Studies with isolated hepatocytes of different species, including man

Nijmegen, 17 januari 1986. J.M. Neis.

I

Bij de ontwikkeling van methoden voor biologische-monitoring m.b.v. proefdieren moet men zich ervan bewust zijn dat de toepasbaarheid van de ontwikkelde technieken onderhevig is aan interspecies verschillen.

Neis, J.M. et al. (1984) Toxicology, 31, 319-327.

Dit Proefschrift.

II

Voor bestudering van de activering van genotoxische stoffen is toepassing van geïsoleerde hepatocyten te verkiezen boven het gebruik van subcellulaire fracties van de lever, zoals het veel gebruikte 9000 x*g* supernatans (S9-fractie).

Dit Proefschrift.

III

Gezien de sterk remmende werking van dimethylsulfoxide (DMSO) op de mutageniteit van o.a. N-nitrosodialkylamines, dient het gebruik van dit oplosmiddel in mutageniteitstesten zoveel mogelijk te worden vermeden.

Mori, Y. et al. (1985) Mutation Research, 142, 153-158.

Dit Proefschrift.

IV

Kankerverwekkende "peroxisome proliferators", waaronder meerdere serumlipidenverlagende stoffen, moeten op grond van het voorgestelde werkingsmechanisme gezien worden als een aparte klasse van chemische carcinogenen.

Reddy, J.K. and D.L. Azarnoff (1980) Nature, 283, 397-398.

V

Bij de teratogeniteitsproblematiek wordt in verhouding te weinig aandacht geschonken aan schadelijke invloeden tijdens de preconceptionele fase als oorzaak voor teratogene effecten.

VI

Het steeds verder verhogen van de gevoeligheid van geavanceerde analytische technieken om exposities aan toxische verbindingen te detecteren getuigt niet altijd van realiteitszin.

VII

De hardnekkigheid waarmee de D-glucaarzuurbepaling in urine telkens weer wordt aangeprezen als lever-enzyminductietest is onverklaarbaar en mist iedere wetenschappelijke onderbouwing.

Brouwer, E. (1985) LAB/ABC, 11, 28-32.

Notten, W.R.F. (1975) Alteration in the D-glucuronic acid pathway and drug metabolism by exogenous compounds. Proefschrift, K.U. Nijmegen.

VIII

De toenemende vraag naar *niet-rokers* in contactadvertenties kan gezien worden als een eigentijdsverschijnsel waardoor de bewustwording van de gezondheidsrisico's van het *passief-roken* wordt onderstreept.

De Volkskrant, 19 november 1985, Rubriek: Dag In.

Bos, R.P. and P.Th. Henderson (1984) Reviews on Environmental Health, 4, 161-178.

IX

De mogelijke aanwezigheid van verontreinigingen van prokaryotische afkomst in produkten vervaardigd via de biotechnologie vraagt grondig toxicologisch onderzoek.

X

De paleolitische voeding zou een voorbeeld functie moeten vervullen binnen de moderne voedingsleer.

Eaton, S.B. and M. Konner (1985) The New England Journal of Medicine, 312, 283-289.

XI

De eerste bacteriën (met een flagella in de vorm van een rechtsgedraaide helix) waarvan werd ontdekt dat ze zowel voorwaarts als achterwaarts kunnen bewegen door de draairichting van de flagella te veranderen, zijn niet de door *Spencer et al. (1985)* als zodanig genoemde *Halobacterium* species, maar *Claustobacter crecentus.*

Spencer, M. (1985) Nature, 313, 183-185.

Koyasu, S. et al. (1985) Nature, 314, 20.

XII

9-Anthryldiazomethaan blijkt een zeer bruikbaar reagens voor de "fluorescent labeling" van carboxylgroepen in aminozuren.

Tsuguchika, Y. et al. (1985) Journal of Chromatography, 348, 425-429.